

Inhaled aztreonam lysine for the treatment of acute pulmonary exacerbations of cystic fibrosis

Thesis submitted in accordance with the requirements of the University
of Liverpool for the degree M.D.

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Declaration:

No portion of the work referred to in this dissertation has been submitted in support of an application for a degree qualification of this or any university or institute of learning. The research in this thesis was conducted at Liverpool Heart & Chest Hospital NHS Foundation Trust and the Institute of Infection & Global Health, University of Liverpool. The thesis author, Freddy Frost (FF) was the principle investigator on all research presented herein, and was supervised by Professor Craig Winstanley, Dr Joanne Fothergill and Professor Martin Walshaw. Individual contributions to the research within this thesis are presented below:

- The AZTEC-CF study was conceived by Dr Dilip Nazareth, who obtained investigator supported research grant funding from Gilead Sciences.
- FF designed the study protocol, patient documents, consent forms and obtained local, ethical and regulatory approvals.
- FF recruited, consented, enrolled and randomised the vast majority of participants with support by Dr Dilip Nazareth and the Respiratory Research Nurses at Liverpool Heart & Chest Hospital NHS Foundation Trust.
- FF or occasionally the Respiratory Research Nurses collected study samples, recorded AEs and completed CRF.
- Spirometry and blood tests were performed in the local NHS accredited laboratory.
- Study sample processing was performed by FF or occasionally staff at the Research Laboratory Liverpool Heart & Chest Hospital NHS Foundation Trust.
- Quantitative culture was performed by Nahida Miah at the University of Liverpool.
- Quantitative PCR was performed by Dr Laura Wright at the University of Liverpool.
- FF performed DNA extraction for all study samples and controls for sequencing.
- Sequencing and pre-processing of raw sequenced data was performed by Dr Gregory Young and Professor Darren Smith at NU-OMICS, University of Northumbria.
- All data handling and statistical analyses including bioinformatic analysis of 16S rRNA sequenced data was performed by FF with support from Gregory Young.

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Abstract

Introduction

Pulmonary exacerbations cause significant morbidity in people with cystic fibrosis, but their treatment with extended courses of intravenous antibiotics may result in important systemic side-effects, adverse reactions and complications. Treatment through the inhaled route, where the lungs are targeted directly with less systemic exposure may be more appropriate, however little is known about the clinical and microbiological utility of using inhaled antibiotics in the acute setting. A recent expansion in available inhaled antibiotics licensed for the chronic suppression of *P. aeruginosa* means some may now also be repurposed in the acute setting. This thesis examined the clinical and microbiological outcomes of using inhaled aztreonam lysine (AZLI) in the treatment of acute pulmonary exacerbations of cystic fibrosis.

Methods

Adults with CF were recruited to an open-label pilot randomised crossover study at a regional adult centre in the UK (AZTEC-CF Study, *ClinicalTrials.gov*: NCT02894684). Inclusion criteria included age > 16 years, *P. aeruginosa* infection and no prior use of AZLI. Exclusion criteria included *Burkholderia cepacia* complex infection and solid-organ transplant. During two consecutive exacerbations requiring hospitalisation for intravenous antibiotics, subjects received 14 days AZLI plus intravenous colistimethate (AZLI+IV) or standard dual intravenous antibiotics (IV+IV). Primary outcome was change in % predicted FEV₁ (ppFEV₁) at 14 days. Key secondary outcomes included health-related quality of life outcomes, sputum bacterial load and 16S rRNA sequenced microbiome dynamics associated with each treatment.

Results

Sixteen people with CF were consented and randomised, and 28/32 (87.5%) exacerbations were completed. At 14 days, improvement in ppFEV₁ was greater in the AZLI +IV compared to the IV+IV arm (mean 13.5% versus 8.8%; paired differences [95% CI] +4.6% [2.1 to 7.2], p=0.002). The minimum clinically important difference in

CFQ-R Respiratory Domain was achieved more frequently in exacerbations treated with AZLI+IV (83.3% vs. 43.8%, $p=0.04$). No significant differences were found between treatments for changes in sputum bacterial load, systemic inflammation, antimicrobial resistance or adverse events. IV+IV and AZLI+IV exerted different effects on the lung microbiome, where IV+IV reduced the abundance of the anaerobes *Prevotella melaninogenica* and *Veillonella dispar*. Comparatively, AZLI+IV was associated with little change in either structure or composition of the microbiome. Neither treatment consistently reduced the abundance of *P. aeruginosa* but where reductions were seen they were associated with improved quality of life.

Conclusion:

AZLI is effective and well tolerated in the treatment of acute pulmonary exacerbations of CF. Superior improvements in lung function and quality of life outcomes alongside a potentially advantageous microbiological profile suggest AZLI may represent a new treatment approach for acute pulmonary exacerbations and larger studies are warranted.

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Commonly used abbreviations

AE	Adverse event
ASL	Airway surface liquid
ASV	Amplicon sequence variance
AZLI	Aztreonam lysine for inhalation
BCC	<i>Burkholderia cepacia</i> complex
CF	Cystic Fibrosis
CFQ-R	Revised Cystic Fibrosis Questionnaire
CFRD	Cystic fibrosis related diabetes
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit
CI	Confidence interval
CRP	C-reactive protein
FEV1	Forced expiratory volume in 1 second
IQR	Interquartile range
IV	Intravenous
LES	Liverpool epidemic strain
LHCH	Liverpool Heart & Chest NHS Foundation Trust
LIS	Levofloxacin inhalation solution
MIC	Minimum inhibitory concentration
NGS	Next generation sequencing
OTU	Operational taxonomic unit
PCA	Principle components analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PIME	Prevalence Interval for Microbiome Evaluation
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal RNA
SAE	Serious adverse event
SD	Standard deviation
TDS	Thrice-daily dosing
TNS	Tobramycin nebuliser solution
WCC	Serum White Cell Count

Chapter 1: Introduction and Review of the Literature

1.1 Genetics of cystic fibrosis

Cystic fibrosis (CF) is the most common life limiting genetic condition in the United Kingdom (UK) with over 10000 people currently living with the disease. [1] CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene located on the long arm of chromosome 7. [2] This gene codes for the CFTR protein, a symmetrical 1480 amino acid structure which functions predominantly as an anion channel and belongs to the ATP-Binding Cassette (ABC) Transporters family of membrane-spanning proteins. [3]

Over 2000 CFTR mutations have been identified with missense and frameshift mutations the most prevalent (39.3% and 15.7% respectively). [4] Mutations can be classed according to the specific mechanism by which they interrupt the synthesis or function of the CFTR protein, see Table 1.2.1. In Europe, approximately 87% of people with CF carry at least one Class II mutation, 17% Class I, 3.9% Class III, 3.3% class IV and 3.0% class V. [5] Geographical variation exists for each mutation class, for example Israel has a higher prevalence of Class I mutations (45%) and people with CF in the UK and Ireland have higher prevalence of Class III mutations (6.2% and 13.9% respectively). [5] The most common mutation is Phe508Del (c. 1521_1523delCTT) with prevalence in the UK CF population of 91% (50.2% homozygous), and only 9 other mutations have a prevalence of >1%, see Table 1.2.2. [1] The F508D mutation is a Class II mutation, causing CFTR protein misfolding resulting in failure of the protein to be localised to the cell membrane. [6]

CF displays autosomal recessive inheritance thereby requiring both parents to carry at least one pathogenic CF allele and the child to receive both in order for the disease to manifest itself. The fact that until relatively recently CF was a lethal condition in early childhood has led to the postulate that carrier status must convey an evolutionary selective advantage to allow CFTR mutations to maintain their frequency in the general population. It has been suggested that carriers of a single

mutated CFTR allele may be less susceptible to secretory diarrhoeas such as seen in *Vibrio cholerae* (cholera) and *Salmonella typhi* infections. [7,8] In the case of cholera, a reduction in anion secretion in response to the cholera toxin with subsequent reduction in colonic fluid loss has been seen in murine carriers of a single pathogenic mutation, however this has not been replicated in humans. [9] *Salmonella typhi* is purported to enter cells via open CFTR channels and CFTR mutations may therefore reduce susceptibility to typhoid fever. [7] Interestingly, the incidence of *Mycobacterium tuberculosis* (TB) has also been reported to be lower in CF carriers and the European TB pandemic of the 17th century may have provided enough historical selective pressure to account for the incidences of CFTR mutations seen today. [10,11]

1.2 Epidemiology

The carrier rate for a CFTR gene is approximately 1 in 25, and while live-birth rates vary from country to country the UK incidence is 1 in 2400 live births. [12] Since the advent of a UK-wide new-born screening programme, over 95% of CF diagnoses are now made in the first year of life. [1]

CF was first distinguished from coeliac disease in 1938, when mucous plugging of the pancreas was noted at autopsies of malnourished young children. [13] Life-expectancy for a new-born with CF at that time was often no more than six months but since then increased recognition of the disease, understanding of its genetic basis and advances in disease management have heralded dramatic increases in survival. Children born with CF today have a median life expectancy of 47 years. [1]

Table 1.2.1 Traditional classification of CFTR mutations. Adapted from De Beck and Amaral [14]

Class	I	II	III	IV	V	VI
Defect	No protein synthesised	No trafficking of protein to cell surface	Impaired gating	Reduced conductance of anions	Functional CFTR but reduced quantity	Functional CFTR in correct quantity but unstable protein
Example	G542X	F508D	G551D	R117H	3272-26A->G	120del123
Disease severity	Severe disease			Less severe disease		

**Table 1.2.2: CFTR mutations in the UK CF population with prevalence >1%.
Adapted from UK CF Registry Report 2016 [15]**

Mutation Name	N	%
F508D	9035	89.7
G551D	581	5.8
R117H	587	5.8
G542X	356	3.5
621+G->T	258	2.6
N1303K	159	1.6
1717-1G->A	157	1.6
1898+1G->A	140	1.4

1.3 Pathophysiology

1.3.1 Ion transport defects associated with cystic fibrosis

CFTR functions as an anion channel and is expressed in many epithelial cells, in multiple organ systems throughout the body and regulates the conductance of both chloride (Cl^-) and bicarbonate (HCO_3^-) anions out of the cell. ABC proteins can also often function as local regulators and CFTR has been shown to regulate neighbouring epithelial Na^+ channels (ENaC). [16,17] CFTR dysfunction therefore results not only in deficient Cl^- and bicarbonate (HCO_3^-) secretion but also drives excess absorption of sodium, thus dramatically perturbing the normal osmotic conditions at the epithelial surfaces. [17,18] The abnormally dehydrated epithelial environment is the underlying aetiology for the majority of clinical sequelae in CF.

1.3.2 Clinical manifestations of CF

The expression of CFTR across a wide range of epithelial cells results in CF being a truly multi-organ disease. Dehydrated epithelial surfaces trigger a milieu of stasis, infection and inflammation which manifest most obviously in the lungs with chronic suppurative lung disease but can affect almost any system in the body.

One of the most common extra-pulmonary manifestations of disease is in the reproductive system where ~98% of males are infertile and subfertility is also well recognised in women. [19] In males, infertility is usually secondary to aberrant development of the vas deferens resulting in congenital bilateral absence of the vas deferens (CBAVD), although semen abnormalities including azospermia, reduced semen volume and abnormal semen pH have also been reported. [19,20] In women with CF, mucus plugs may act as mechanical barriers to conception but the reproductive tract is otherwise structurally normal. [21]

The pancreas is another commonly affected organ and the term “cystic fibrosis” was originally a description of pancreatic lesions seen during autopsy. [22] Bicarbonate secretion is particularly important in the healthy pancreas where it buffers various

digestive acids but in the CF pancreas a lack of epithelial bicarbonate contributes to pancreatic injury as viscid unbuffered acidic secretions do not flow to the bowel and instead cause localised pancreatic damage. [23] Progressive local injury leads to pancreatic fibrosis with subsequent exocrine and endocrine insufficiency. Exocrine abnormalities are characterised by malabsorption of fat and also fat-soluble vitamins resulting in malnutrition. Endocrine dysfunction develops when damaged, dysregulated *Beta*-cells struggle to release insulin adequately and cystic fibrosis related diabetes (CFRD) ensues. [24] CFRD is not only associated with aberrant glycaemia, but also carries with it significant morbidity and a three-fold increase in mortality. [25]

Elsewhere, stasis of hyperviscous bile can accumulate in the biliary tree causing focal inflammation and fibrosis. In this setting persistent hepatocyte injury can lead to CF related liver disease, the most serious consequence of which is decompensated cirrhosis, the third most common cause of death in children and adolescents with CF. [26,27] Other common co-morbidities include polyposis and chronic sinusitis in the upper respiratory tract, low bone mineral density, gastro-oesophageal reflux and renal disease. [28–31]

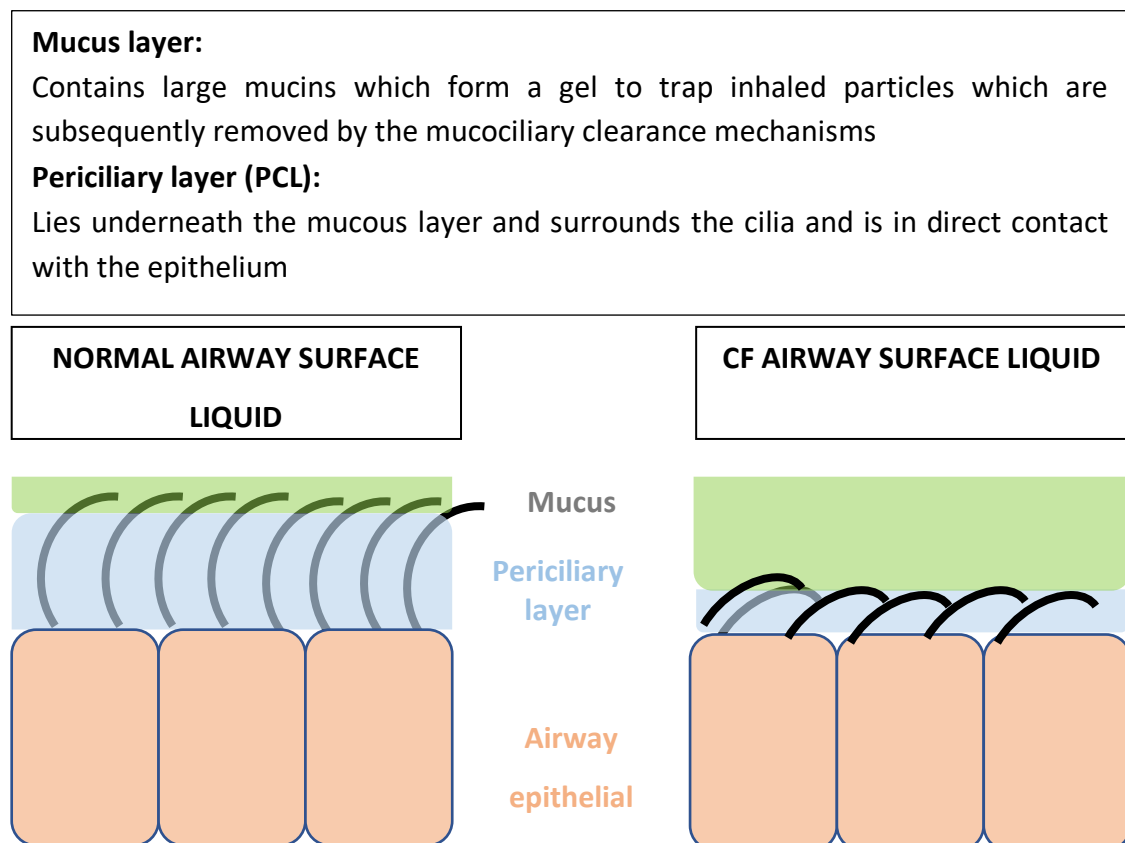
1.3.3 Mucociliary consequences of impaired CFTR function

Despite the prevalence of co-morbidities in multiple other systems, the single biggest impact on morbidity and mortality in people with CF is lung disease, which develops and progresses as a direct pathophysiological consequence of a dehydrated airway surface liquid (ASL), see Figure 1.3.1. [32] The ion transport defects outlined in section 1.3.1 disrupt the osmotic composition of the periciliary layer (PCL), which becomes progressively compressed and increasingly viscous such that the ciliary clearance mechanisms slow down and eventually stop. [33] Mucus stasis ensues creating a niche for infection and inflammation. [34]

Further compounding the local environmental disturbance is reduced HCO_3^- which appears to be important for the release and efficient expansion of mucins within the ASL. [35,36] HCO_3^- has long been recognised as a buffer in the pancreas but has also

more recently been implicated as playing a similar role in the airways where abnormally acidic pH was found in cultured CF human bronchoepithelial cells as well as CF pigs. [37,38] The reduced pH noted in CF pigs was associated with reduced bacterial killing by the innate antimicrobial peptides usually resident in the ASL. Thus, introducing the concept of impaired innate immune defence in CF.

Figure 1.3.1: Airway surface liquid in normal and CF airways



1.3.4 Impaired innate immune defence in CF

The lungs have many protective mechanisms against the constant exposure to inhaled environmental pathogens and irritants. One such mechanism is an array of antimicrobial peptides found in the ASL that are able to rapidly kill or inactivate inhaled bacteria.[39,40] These negatively charged cationic peptides disrupt bacterial membranes and are found in the ASL where they are active against a wide range of bacteria including the CF pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa*. [39,41] However, attachment and subsequent disruption of the bacterial membrane is dependent on an alkaline pH and the acidified CF ASL impairs their activity. [38,39,42]

Alongside the innate antimicrobial properties of the ASL, another powerful defence mechanism is the recruitment of white blood cells (e.g. neutrophils) into the airways. Neutrophils migrate into the lungs via the post-capillary venules in response to complex signalling from cytokines and chemoattractants. [43] Once in the airways, bacterial phagocytosis occurs accompanied by the release of proteases to degrade by-products of phagocytosis. Excess accumulation of proteases results in an imbalance of innate inhibitors e.g. alpha1-anti-trypsin, such that proteolytic activity is exerted on the CF airway itself, degrading structural proteins e.g. collagen and elastin. This results in prolonged and sustained inflammatory airway damage. [44,45] The most well-characterised of the proteases is neutrophil elastase (NE), concentrations of which are higher in the airway of people with CF compared to those with non-CF bronchiectasis and also healthy controls. [46] In CF, increased NE has been associated with poorer clinical outcomes including increased bronchiectasis and airway inflammation in childhood, and accelerated lung function decline in adulthood. [47,48] Bacterial burden is high in the CF airways, but even allowing for that, neutrophil recruitment is exaggerated and disproportionately prolonged. [49,50]

The exaggerated accumulation of neutrophils in the CF airway has been suggested to be related to the presence of CFTR on neutrophils themselves. [51] Indeed, CFTR

expression has been confirmed on the membranes of neutrophils and reduced expression is noted in CF. [52] CFTR defective neutrophils appear to have reduced bacterial killing properties but also delayed death, perhaps explaining the observed accumulation in the airways. [53] Also, recent evidence suggests that the CF neutrophils are more likely to form neutrophil extracellular traps (NETs), web-like traps formed by dying neutrophils. NETs have the ability to immobilise and kill bacteria but can conversely induce excess inflammation, particularly when pathogens such as *P. aeruginosa* are able to adapt to resist NET-mediated killing. [53–55]

1.3.5 Pulmonary infection in CF

The defective mucociliary clearance and impaired innate immunity discussed earlier result in a pulmonary microenvironment that is susceptible to chronic infection and subsequent progressive irreversible lung damage which remains the leading cause of morbidity and mortality in CF. [32]

The original description of CF identified tubular bronchiectasis with purulent bronchitis and bronchopneumonias on autopsy and also reported *S. aureus* was the most common organism cultured. [22] *S. aureus* remains the most commonly isolated organism in younger patients with CF, but as age increases the microbiology of CF changes and by adulthood *P. aeruginosa* is the most prevalent pathogen. [56,57] Other well recognised CF pathogens include species of the *Burkholderia cepacia* complex (BCC), some of which are associated with accelerated pulmonary decline. [58] More recently other microbes such as *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and non-tuberculous *Mycobacteria* (NTM) have been termed “emerging pathogens” due of their increasing prevalence, although the pathogenicity of some remains controversial. [59–62]

P. aeruginosa, a Gram-negative facultative anaerobe found ubiquitously in the environment, remains the major pathogen associated with CF with a prevalence of over 50% in adults. [1] As an opportunistic pathogen it is the second most common

cause of hospital acquired pneumonia, often in immunocompromised hosts, accounting for 8% of all health-care associated infections in the US. [63] *P. aeruginosa* has the potential to be resistant to multiple antibiotics and the World Health Organisation recently designated it as one of only three “Critical” pathogens requiring urgent research and high-priority development of new antibiotic approaches. [64]

In CF, *P. aeruginosa* infection is associated with accelerated pulmonary function decline and is the leading cause of morbidity and mortality. [1,32,65] *P. aeruginosa* is most commonly acquired from the environment but transmission of “epidemic” strains of *P. aeruginosa* between people with CF is also well documented. [66] Transmissible strains were first identified based on molecular analysis in the 1990s and some strains such as the Liverpool Epidemic Strain (LES) have been associated with even poorer prognosis. [67]

Despite the changes to the CF lung environment discussed earlier, the lung is still a hostile and challenging environment for microbes due to factors such as osmotic gradients, oxidative stress, the presence of other bacteria and an exaggerated host response. [68] A large genome allows *P. aeruginosa* to undergo a number of adaptations and this versatility is a trait that allows it to overcome the challenges of colonising CF lung.

Although the precise sequence of events that take place between inhalation of *P. aeruginosa* and infection being established is not known, an initial step is likely to be adhering to a surface in the lungs. Healthy lungs have a functioning mucociliary escalator to help clear any inhaled pathogens/irritants but the static, viscous secretions in CF airways are full of mucins to which *P. aeruginosa* can bind and become enveloped within. [69] Despite adequate ventilation of the airways themselves, hypoxic zones exist within the large aggregations of luminal mucous and eventually *P. aeruginosa* is enveloped within these oxygen-deplete aggregations and can start to adapt by slowing its growth and overproducing alginate. [69–71]

The overproduction of alginate is the hallmark of an adaption from a free-swimming planktonic phenotype to the “mucoid” phenotype. Alginate, a cationic exopolysaccharide, provides a structure for a biofilm matrix allowing microbial cells to be held together in an organised community, thereby providing physical protection from stressors such as antibiotics and host immune cell phagocytosis. [68] The mucoid phenotype and biofilm growth are associated with greater pathogenicity in CF, probably at least in part due to reduced ability of the innate immune system and antibiotics to penetrate and overcome the infection. For example, transition to mucoid phenotype has been associated with immediate pulmonary consequences including reduced lung function, increased symptoms and changes on chest radiography. [72] In the longer term, a mucoid phenotype is associated with increased bronchiectasis, accelerated pulmonary function decline and increased mortality. [73,74] Mucoid phenotype and biofilm formation are therefore considered evidence of a transition from acute to chronic *P. aeruginosa* infection in CF.

Other phenotypic adaptations include increasing antibiotic resistance, [75,76] loss of motility, [77] the adoption of a hypermutator role, [78] DNA repair, [76] and iron acquisition. [79,80] Together these traits are termed pathoadaptations. [68] Pathoadaptations occur as a result of mutations in genes for key regulatory proteins and are often evidence of selective pressure from the lung environment. Some mutations acquired in key regulatory genes are consistently found in different patients suggesting parallel evolution during the process of adaptation, however this is not always the case and there may be different trajectories of evolution between individuals. [68,70,81,82] More recently dissection studies of explanted lungs have confirmed that even within the same patient, genetically and phenotypically distinct populations are found in different geographic regions of the lung perhaps as a result of the different regional environmental conditions and subsequent distinct evolutionary trajectories. [83]

1.4 Pulmonary exacerbations

1.4.1 Definition

One of the hallmarks of CF pulmonary disease is episodic acute worsening of symptoms and lung function. These events are termed pulmonary exacerbations. Although the definition of an exacerbation not universally agreed, there are two broad approaches to defining these important events:

1. A requirement for treatment with acute antibiotic therapy
2. A cluster of symptoms or signs that indicate a recent deterioration.

The first approach has often been used in clinical studies however there exists considerable inter-clinician, inter-centre and inter-country variation in the thresholds for initiating antibiotics. For example, some centres only utilise IV antibiotics in acute deteriorations whereas others have adopted a strategy of giving IV antibiotics at regular intervals, e.g. 3-4 monthly regardless of clinical status. [84–86]

Given the heterogeneity in the first approach, attempts have been made to standardize the definition into a scoring system or a set of minimal criteria. [87,88] Fuchs defined exacerbations as a clinical need for IV antibiotics in the presence of at least 4 out of 12 possible signs or symptoms. [87]A EuroCareCF working group simplified this approach and defined an exacerbation as the clinical need for acute treatment in the presence of two of the following symptoms/signs.

- Change in sputum volume or colour
- Increased cough
- Increased malaise, fatigue or lethargy
- Anorexia or weight loss
- Decrease in pulmonary function by 10% or more / Radiographic changes
- Increased dyspnoea [88]

1.4.2 Pathogenesis

Despite the clinical significance of acute pulmonary exacerbations, their pathogenesis remains incompletely understood. A number of different aetiologies and mechanisms have been suggested to be responsible including bacterial infection, viral triggers, fungal infection, inhaled pollutants and even proton pump inhibitors. [89–93] With regards to microbiology, acute pulmonary exacerbations do not tend to be associated with the acquisition of new bacterial species or even new clones of an already present species. [94] Sputum bacterial load does not appear to increase prior to acute pulmonary exacerbations, leading to speculation that acute pulmonary exacerbations may represent spread of already present infection to previously undiseased areas of the lung. [91,95,96] The triggers for spread to new areas may be multifactorial. For example, viruses such as Respiratory Syncytial Virus (RSV) have been demonstrated to promote adherence of *P. aeruginosa* to epithelial cells and hence an initial viral insult to an uninfected area of lung could result in adherence of *P. aeruginosa* and subsequent infection in that area. [97] Equally, an imbalance in an established regional population of bacteria within the lung may result in a more active sub-population, which could in-turn drive a spread of planktonic bacteria to new areas of lung. The resulting host response would elicit the symptoms and signs of an acute pulmonary exacerbation and the newly migrated planktonic population would be more sensitive to antibiotics, hence explaining the response to antibiotics. [95]

An alternative hypothesis to the spread of existing infection is the concept that changes occur within the already established infection and these changes drive an increased localised immune response. An example of this would be the phenotypic shift towards an increase in pyocyanin overproduced observed at exacerbation. [98] Any within-population phenotypic shifts as a result of selective pressures in the local environment would result in a similar sputum bacterial load but a resultant change in metabolites, virulence factors or alginate production could trigger increased immune cell recruitment to the area with subsequent excess protease release and

development of the symptoms and signs of an exacerbation with resultant lung injury. Much of the research investigating exacerbations has to-date focussed on single bacteria or host-bacteria interactions. More recently, there has been increasing understanding of the polymicrobial nature of CF lung infections, much of which has been garnered by novel culture-independent techniques such as next generation sequencing (NGS).

1.4.3 Impact

Whilst there has been debate regarding the definition and pathogenesis of exacerbations, there is consensus when it comes to the clinical significance of these acute events. Acute pulmonary exacerbations are an important driver of morbidity in CF and have been associated with reduced quality of life outcomes, sleep disturbances and impaired neurobehavioural function. [99,100] Moreover, severe exacerbations requiring IV antibiotics are themselves a risk factor for further exacerbations as well as an independent risk factor for mortality. [101,102] A number of studies have demonstrated lung function frequently does not return to baseline after exacerbations and progressive loss of lung function from sequential exacerbations can account for half of the lung function deterioration seen in people with CF. [103–105] The healthcare impact is also considerable with a US study estimating each exacerbation requiring IV antibiotics to cost in the regions of \$36000. [106]

1.5 The Lung Microbiota in Cystic Fibrosis

Traditional culture techniques rely on growing bacteria on media in laboratory conditions often optimised for growth of specific organisms to allow subsequent identification. In the last 20 years, novel techniques utilising NGS to identify bacteria have become available, enabling detection and description of bacterial communities without the need for conventional culture. These technologies have allowed a greater understanding of bacterial communities throughout the human body and have revealed functional roles in both health and disease.

A healthy human gut for example is home to a highly diverse community of microorganisms, which has symbiotic functions including metabolism of otherwise indigestible compounds and defence against opportunistic pathogens. [107,108] Furthermore, bacteria in the gut influence the stimulation and development of the innate mucosal immune system. In addition to the roles in health, there has been significant interest in the relationship between microbiomes and diseases such as obesity, inflammatory bowel disease and diabetes mellitus. [109–111]

In the lungs, studies utilising culture-independent techniques have identified the presence of bacterial communities much more complex than previously appreciated. The lungs were long considered to be an inherently sterile environment, in part due to the fact that conventional culture techniques often yielded negative results during health and it was only during disease that pathogens were detected.[112,113] However, the advent of culture-independent techniques has demonstrated that multiple organisms comprise a community in the lungs regardless of health status. [114–116] [250-252] This community is termed the lung “microbiota”, a term which is often used interchangeably with “microbiome” although the two terms are subtly different, see Table 1.5.1. In this section I discuss the techniques employed and evidence generated so far in relation to the cystic fibrosis lung microbiome.

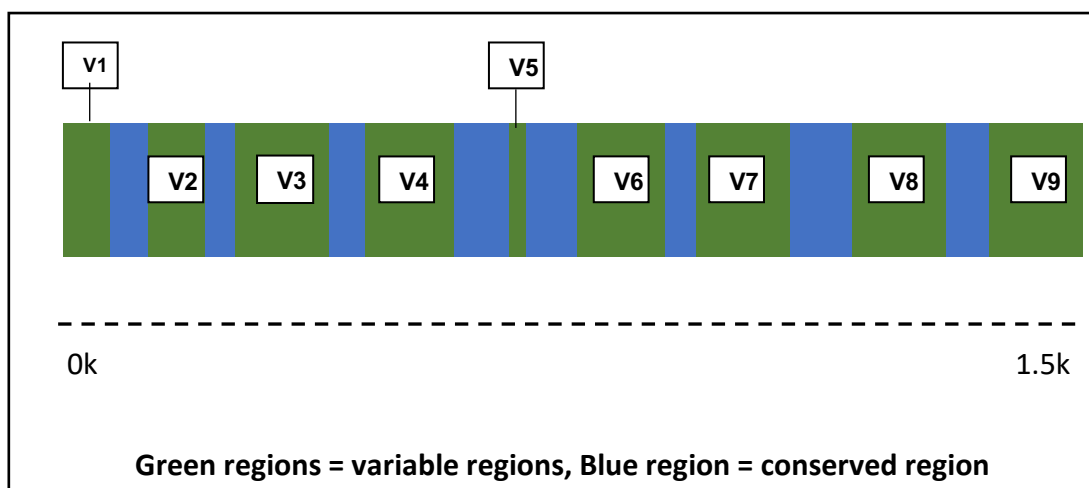
Table 1.5.1: Glossary of terms and definitions

Term	Definition
Microbiome	The collection of genomes from all the microorganisms in a particular environment
Microbiota	The microorganisms found in a particular environment
16S rRNA gene	A gene which codes for a ribosomal subunit. Present in all prokaryotes and has variable regions, which differ slightly between bacterial species
Alpha diversity	Within-sample diversity
<u><i>Commonly used alpha diversity metrics</i></u>	
Richness	A measure of the number of species in a community
Evenness	A measure of similarity of the relative abundance for each species in a community. I.e. Does one species dominate or do all species have similar relative abundance
Shannon Diversity	Combination of richness and evenness.se
Beta-diversity	Between sample diversity
Operational taxonomic unit (OTU)	Clusters of similar sequences assumed to represent a taxonomic unit of a bacterial species or genus.
Amplicon sequence variant (ASV)	Inferred DNA sequence representing a true taxonomic unit of a bacterial species or genus

1.5.1 16S rRNA gene sequencing

The 16S rRNA gene codes for a ribosomal subunit present in nearly all bacteria. The gene itself is approximately 1.5kb long and consists of conserved regions, similar in nearly all microorganisms, and nine variable regions labelled V1-V9, which are practically specific to each microorganism, see Figure 1.5.1 . [117] The identification of the precise DNA sequence of a bacteria's 16S rRNA gene therefore allows inference of its identity in a similar way that a unique fingerprint allows identification in humans.

Figure 1.5.1: Schematic illustration of structure of the 16S rRNA gene



In order to deduce the 16S rRNA gene sequence, DNA is extracted from a sample, e.g. sputum, and the 16S rRNA gene is amplified using polymerase chain reaction (PCR). Next generation sequencing of the amplified sequences allows elucidation of the precise gene sequences and estimation of their relative abundance. Online reference databases can then be used to match each sequence to an organism to allow identification of each member of the polymicrobial community. However, it is important to note that sequencing of the 16S rRNA gene has limited resolution and often cannot distinguish between individual species with similar 16S rRNA gene sequences. Instead of distinct species, sequences are referenced against and assigned into operational taxonomic units or more recently amplicon sequence variants.

1.5.2 Amplicon sequence variants vs. operational taxonomic units

As described above, 16S rRNA gene sequencing often cannot distinguish between individual species because many very similar species share similar 16S rRNA gene sequences. Intrinsic errors in the amplification or sequencing process can result in amplified sequences differing from each other very slightly making species differentiation even more challenging. [118,119] To date most studies have overcome this by clustering very similar sequences together into operational taxonomic units (OTU). An arbitrary threshold of 97% is commonly used for clustering such that sequences differing from each other by less than 3% are considered to be the same OTU. OTUs can then be matched against known OTUs in reference database to provide taxonomic information, often providing resolution to the genus level.

More recently, a new approach to taxonomically resolve sequences has been developed which attempts to limit the variation caused by intrinsic amplification or sequencing errors. This approach infers the biological sequences within a sample using a model which relies on input read abundances (true reads are likely to be more abundant) and distances (less abundant reads only a few base-differences away from a more abundant sequence are likely error-derived). The sequences identified in this

technique are termed 'amplicon sequence variants' (ASV) and replace OTUs. [118,120] ASVs hold a number of advantages over OTUs; firstly, by eliminating amplification/sequencing error, more sequences can be accurately resolved to the species level which has clear benefits for microbiome research. Secondly, an ASV is a fixed, defined sequence and can therefore be consistently labelled allowing comparison between studies. Again, this holds obvious advantages to microbiome researchers. With these advantages in mind there have been calls for ASVs to replace OTUs as the basic unit in 16S rRNA gene sequencing studies. [120] Most CF microbiome research to date predates these calls and therefore OTUs form the basis of most studies described in this chapter.

1.5.3 Ecology of the microbiome

Given the large number of species identified even in healthy lungs, ecological theory and analyses are often employed to understand community dynamics. According to ecological principles the composition of the lung microbiome is determined by three factors:

1. Immigration of organisms into the lung
2. Elimination of microbes from the airways
3. Regional growth factors [113]

The lung microbiota in healthy individuals is dictated largely by immigration and elimination and hence generally consists predominantly of those Gram-negative anaerobes also resident in the oral flora such as *Prevotella spp.* and *Veillonella spp.* [113] In disease, the regional growth conditions are altered and niches for other species to thrive are created. In CF, viscous secretions, altered pH, nutrient availability and architectural disturbance may all help select for a community of altered composition to that of healthy individuals.

Ecological diversity analyses are frequently used to describe and quantify changes within the lung microbiome. The diversity within a single sample is known as alpha

diversity, whereas diversity between samples is known as beta-diversity. [121] A description of frequently used diversity metrics are included in Table 1.5.1. Longitudinal changes in diversity over time, and in response to the intensive antibiotic treatment that people with CF are exposed to, has been the subject of much interest in the last decade.

1.5.4 CF respiratory microbiota in early life

Understanding the development of the CF respiratory microbiota in early life has attracted interest in order to appreciate the driving factors behind the distinct microbiota seen later in life and also to identify potential opportunities for intervention. Neonates and infants cannot expectorate sputum independently and bronchoalveolar lavage (BAL) is only used sparingly; hence, upper respiratory tract samples are often used as surrogates. This approach is imperfect given discordance between BAL samples and upper respiratory tract (URT) samples has been observed for some taxa. [122] Nevertheless, concordance is high for some important taxa such as *Moraxella* and *Staphylococcus*, and in the absence of less invasive techniques URT sampling enables early estimation of the neonatal lower respiratory tract.

The composition of CF nasopharyngeal microbiota diverges from that of non-CF infants as early as the first few months of life. [122,123] Newborn healthy infants appear to have nasopharyngeal microbiota dominated by *Moraxella* spp., *Corynebacterium* spp. and *Haemophilus* spp., a community structure that persists for at least the first 6 months of life. Conversely the CF nasopharynx microbiome is initially dominated by *S. aureus* before a gradual increase in *Streptococcus* spp. and *Moraxellaceae* at 3 months of age. [123] Despite the increased *S.aureus* seen in CF, there were no decreases in measures of richness or diversity indicating that changes are due to differing microenvironment rather than inter-species competition. [124]

The divergence observed in the first few months of life precedes antibiotic administration and demonstrates that CF itself is associated with compositional

changes in the microbiota, but as CF infants grow older exposure to antibiotics, either via acute treatment for respiratory illnesses or prophylaxis against classic CF pathogens, becomes inevitable. Mika *et al.* investigated the relationship between antibiotics and the nasal microbiota by following 30 newborn infants with CF and performing fortnightly sampling for the first 12 months of life. [125] Antibiotic administration was associated with an increase in the Shannon diversity measure (a measure of the richness and evenness of a community) but this was judged to be most likely secondary to an increase in transient colonisers. Interestingly, antibiotic therapy was staphylococcal directed but decreases in *Staphylococcus* OTUs were not seen. Instead significant reductions in *Moraxellaceae* were observed and when oligotyping was used to better evaluate changes in *Staphylococcus* at the species level, mild reductions in *S. aureus* were offset by increases in *S. epidermidis*, leading the authors to suggest that *S. epidermidis* may act as a reservoir of resistance. These findings were supported by Prevaes *et al.* who conducted a similar study in a slightly older population (mean age 2yrs old). [123] There, antibiotic treatment was associated with reductions in *Moraxellaceae* and *S. aureus* OTUs, with increases in other staphylococcal OTUs, although more specific oligotyping was not performed.

As children grow older, sampling from the lower airways becomes more common and comparisons between the lower and upper airways become feasible. Given the close proximity and inter-related spaces of the nose, throat and lungs, it could be expected that they share similar community structures, however the reality is that the nasal community appears different from that of the throat and lung, which are much more closely aligned. Boutin *et al.* found differences in the community structure of the nasal cavity compared to throat and sputum samples in that diversity, richness and evenness were significantly higher in nasal samples, and up to 21 of the 76 most abundant nasal OTUs were not present in the throat or sputum samples. [126] Interestingly, the authors also found that people with CF could be broadly defined into one of two ecotypes based on the presence or absence of *Pseudomonas* OTUs, and the similarities between throat and sputum samples began to diminish once *Pseudomonas* was present. Muhlebach *et al.* supported these

findings in a recently published study, which for the first time included routine sequential BAL sampling in young children as part of the large AREST-CF study cohort in Australia and USA. [127] There, lower airway cultures mirrored those of the oral cavity until approximately age 2 years, when increasing predominance of known CF pathogens was observed and communities subsequently diverged. This has a number of clinical implications in that firstly, throat swabs can provide adequate representation of the lower airways in very young children, and secondly prevention or delay of this transition point by manipulation of the microbiota could theoretically be a strategy to improve outcomes later in life.

1.5.5 Progressive loss of diversity

Once the lungs are colonised with CF pathogens a pattern of progressively uneven community structures ensues. Cox *et al.* examined bio-banked sputum samples from a cohort of 63 clinically stable people with CF of ages ranging from 9 months to 72 years. [115] This cross-sectional approach identified loss of community richness, evenness and diversity as age increased. *Pseudomonas* and *Burkholderia* OTUs began to progressively dominate in older people with CF, and the changes in community structure were inversely associated with pulmonary function. In a similar study design with 269 patients, Coburn *et al.* also found sample diversity inversely correlated with age and disease stage. Progressive loss of diversity was particularly correlated with *Pseudomonas* and *Burkholderia* abundance, which notably increased after the age of 25 years. [128]

Zhao *et al.* were the first to confirm these findings longitudinally when they followed 6 patients over a 9-year period with serial sputum collections. [129] It was observed that the 3 patients with what was termed as more “progressive” disease had significant decreases in community diversity over the course of several years. This study was soon followed by Fodor *et al.* who focussed more on changes in the microbiota associated with acute changes in clinical status, but did observe a strong correlation between low species richness and poor lung function. [130] Stokell *et al.* followed a single patient up over 3 years and observed increasing total bacterial load as well as diminishing community richness and diversity. [131]

Contrastingly, Whelan *et al.* published a study of 6 patients who submitted thrice-weekly sputum samples for a year. [132] No overall changes in community structure were observed over the course of the year and the authors concluded that the respiratory microbiome is unique to each patient and the previously reported associations between community structure and clinical parameters may be true on a cohort/population level but not at an individual level. However, it is worth noting that the 6 patients in the study appeared relatively stable with a median of only 1 exacerbation in the 12-month study period and it is likely that the follow up period was not long enough to capture the indolent changes likely to be present in those patients. A much longer study period was adopted by Acosta *et al.*, who analysed samples from patients with bio-banked sputum samples in three discrete historic cohorts spanning 20 years at a single-centre. [133] Across the cohorts, clinical status was better in the more recent cohorts, and this was associated with a decrease in the proportion of *Pseudomonas*-dominated communities and increased overall diversity, further supporting a relationship between community structure and clinical outcomes.

The association reported in most studies between community structure and clinical outcomes has inevitably led to the question of whether a less diverse, less rich or less even microbiome is simply a marker of increased pulmonary disease or is itself a driver in disease pathogenesis. [134] If the latter were true, efforts to promote a more diverse community could have the potential to slow pulmonary disease progression. An Italian group has led efforts to find patterns or signatures in the microbiome that may predispose patients to accelerated lung function decline, however no causal association has been elucidated. [135–137] Instead, Zhao *et al.* found that the relationship between age, lung function and community diversity disappeared once controlled for antibiotic use, suggesting antibiotic therapy is the predominant driver of reducing community diversity. [129] The same group later developed a statistical approach to more precisely correct for antibiotic exposure when examining relationships between microbiota and clinical outcomes. The

approach was applied to 478 sputum samples and confirmed antibiotic use is an independent predictor for decreased diversity. [138]

Accurately recording antibiotic use is troublesome in longitudinal CF studies due to the widespread use of long-term antibiotics for which compliance may be heterogeneous and also frequent episodic use of acute antibiotics in CF (which can often be self-directed). [139] Furthermore, the majority of CF microbiome studies to date have been retrospective in nature, which brings added challenges for establishing concurrent antibiotic use when not accurately recorded at the time.

Pittman *et al.* were able to prospectively perform bronchoscopy and record antibiotic exposure for 32 children with CF as part of the AREST-CF study. In that study community diversity was found to be much lower in the BAL of those patients receiving antibiotics despite all participants being young children with mild lung disease. [140] Thus it appears likely that the strong association between community structure and degree of lung disease is related to the inevitable prolonged and aggressive use of antibiotics in CF, rather than direct pathogenesis from a less diverse microbiome.

1.5.6 Changes associated with chronic suppressive antibiotics

Inhaled antibiotics such as colistimethate, tobramycin, AZLI and levofloxacin preparations are all licenced in the UK for the treatment of chronic *P. aeruginosa* infections and have, to varying degrees, demonstrated improvements in lung function and exacerbation rates as well as sputum density of *P. aeruginosa*. [141–144] However, despite the widespread use of these inhaled anti-pseudomonals in CF, the effect of these treatments on the microbiome remains poorly defined. Furthermore, many patients receive chronic macrolide therapy over many years, at least in part for its immunomodulatory effects, yet similarly little is known about the effects of this potential persistent selective pressure on the microbiota.

When considering inhaled antibiotics there is contrasting evidence as to their influence on the CF microbiome. For example, Kramer *et al.* reported no relationship

between inhaled antibiotic treatments and bacterial community structure (although it is unclear which agents patients were using in that study), whereas a more recent study found chronic alternating inhaled antibiotic therapy to be associated with less diverse microbiomes. [145,146] Acosta *et al.* utilised a prospectively collected Canadian sputum biobank and primarily investigated changes in CF cohort microbiota over time, but also assessed whether different long-term antibiotics were associated with distinct microbiota. [133] 82 samples from 42 patients were sequenced and beta-diversity differed in those receiving long-term inhaled tobramycin and colistimethate, but interestingly no differences were seen in people receiving AZLI. The authors also assessed the impact of long-term oral azithromycin and nebulised dornase-alfa but these agents were not associated with any differences in lung microbiota composition or structure.

Perhaps intrigued by the lack of effect seen for AZLI given its established clinical benefits, the same group has investigated its effects in more detail. The same Canadian biobank was used to identify and sequence 80 samples from 24 people with CF and naive to AZLI followed by 82 samples from the same patients following initiation of AZLI. [147] Overall no differences were observed in alpha or beta diversity measures, but lower relative abundances of *Prevotella* were seen following AZLI initiation. The authors then sub-classified study participants into AZLI “responders” and “non-responders” based on clinical outcomes and found “non-responders” to have lower abundance of *Pseudomonas* and higher abundance of *Staphylococcus* OTUs.

The same study group recently reported results from a multi-centre observational study of long-term AZLI use where sputum samples were taken before, during and after a 28-day AZLI treatment period. [146] In keeping with their previous findings, AZLI was not associated with consistent changes in the microbiota; however changes were present at an individual level. Again, participants were classed as responders or non-responders and a higher relative abundance of *Staphylococcus* and *Streptococcus* OTUs at baseline was associated with a lack of clinical response,

validating some of the findings of the retrospective study. These findings raise the prospect of signatures in an individual's microbiota acting as a biomarker for response to antibiotics.

1.5.7 Changes associated with acute pulmonary exacerbations

Despite the importance of exacerbations on long-term outcomes of people with CF, the pathophysiology of these events remains undefined.[99,148] Clinically, exacerbations are frequent and are characterised by changes in symptoms beyond day to day variation and can include increase in sputum volume or purulence, shortness of breath and fatigue. The precise mechanisms underlying these important events remain elusive and studies looking for answers using culture-independent techniques have not found consistent answers. For example, one may expect to find evidence of increases in known pathogens at the time of exacerbations yet there is no consistent evidence of this. [95] In fact, a number of studies have found the CF microbiota to be extremely stable over time and resilient to change at exacerbation and also following subsequent treatment. [129,130,149,150]

However, when the community structure as a whole is considered, a number of larger studies have found reduced diversity or richness at the times of exacerbation compared to clinical stability. Coburn *et al.* found small decreases in Shannon diversity in exacerbation samples compared to baseline in their study of 269 people with CF and similarly, Filkins *et al.* found that samples taken during exacerbations had significantly lower diversity than samples taken when patients were stable. [128,151] Perhaps most convincingly Li *et al.* collated data from 18 previous studies to analyse over 700 sputum samples and found significant reductions in community richness at exacerbation. [152]

In terms of changes in individual taxa at the time of exacerbation, Carmody *et al.* followed 4 patients for 3 months with daily sputum sampling and observed daily stability between exacerbations but increased *P. aeruginosa* abundance at the time of exacerbation in some patients and increases in *Prevotella* OTUs in others. [153] These findings help introduce two new concepts; firstly, the potential for

exacerbations to appear similar phenotypically but have different underlying aetiology with only some being due to changes that can be observed in the microbiota, and secondly that previously overlooked anaerobes may play a pathogenic role.

The first concept is supported by Whelan *et al.* who found in longitudinal sampling of 6 patients that some but not all exacerbations were associated with changes in the microbiota. [132] In exacerbations of chronic obstructive pulmonary disease (COPD), four distinct aetiological clusters have been identified: Bacterial, viral, eosinophilic predominant and “paucinflamatory”. Even if these clusters are not mirrored in CF, it is plausible that not all exacerbation clusters would be associated with changes apparent in either individual taxa or overall bacterial community structure. [154] Changes in the metabolic activity of specific taxa or the community as a whole triggering an exacerbation could be another explanation for an apparent lack of change in the community structure seen in some studies. The metabolites lactate and putrescine were found by Twomey *et al.* to be increased during exacerbation in the absence of clear changes in the community structure.[155] Quinn *et al.* used ecological functional networking to identify the non-melavonate pathway of isoprenoid synthesis as a “keystone” pathway in CF infections. Intriguingly fosmidomycin, an anti-malarial agent, is known to be effective at targeting this pathway. [156]

The second concept to emerge from the study of Carmody *et al.* relates to the changes in *Prevotella* abundance at the time of exacerbation and raises the prospect that species not considered conventional CF pathogens may play a role in exacerbations. [153] Anaerobic species are easily overlooked in conventional selective culturing due to the requirement for anoxic culture yet are frequently identified in culture-independent analyses of the CF lower airways. In addition to *Prevotella*, *Gemella* and the *Streptococci anginosus (millieri)* group have both been found to have associations with clinical instability. [151,153,157] Anaerobes have been shown to have the potential to modulate *P. aeruginosa* gene expression in the

polymicrobial setting; hence even if not directly pathogenic, they may still play a contributory role to the pathogenesis of some exacerbations. [158,159]

To summarise, the aetiologies underpinning the transition from a stable state to an acute exacerbation are not well understood. It seems likely there are multiple aetiological clusters only some of which may be associated with changes in community structure.

1.5.8 Changes associated with treatment for acute pulmonary exacerbations

Traditional dogma would dictate that intensive, targeted antimicrobial therapy with dual anti-pseudomonal agents will result in significant reductions in abundance of *P. aeruginosa*; however, in a similar vein to the findings from studies of the onset of exacerbations, microbiota responses to treatment for acute pulmonary exacerbations in CF have not aligned with this conventional understanding of acute infections in CF.

One of the predominant themes that has emerged from studies of the effect of acute antibiotics on the lung microbiota is that *P. aeruginosa* is not impacted to the same degree as other members of the community. For example, Daniels *et al.* studied 12 adult with CF across the cycle of an exacerbation, and found that following initiation of anti-pseudomonal antimicrobials, the relative abundance of *P. aeruginosa* actually increased, alongside a reduction in the total number of species detected. [160] Cuthbertson *et al.* also found no evidence of reduced *P. aeruginosa* abundance in a study of 12 CF patients receiving treatment for pulmonary exacerbations. Instead, reductions in *Streptococcus sanguinis*, *Prevotella* and *Porphyromonas* OTUs were observed. [150] Similarly, Li *et al.*, in their analysis of over 700 sputum samples, found that antibiotic treatment had no effect on *Pseudomonas* abundance, but did have significant effects on *Gemella*, *Staphylococcus*, *Acitnomycetes*, *Moraxllaceae* and *Fusobacterium*. [152] Further, Fodor *et al.* again found that dominant taxa such as *Pseudomonas* and *Burkholderia* were unchanged when compared at the beginning

and end of an exacerbation, but the relative abundances of *Gemella*, *Streptococcus* and a small number of other less abundant OTUs were all reduced. [130]

In contrast, two studies have found reductions in *P. aeruginosa* following treatment. Firstly, Zemanick *et al.* investigated the association between inflammation and changes to the airway microbiota during treatment for exacerbations and found that although bacterial load did not change, the relative abundance of *P. aeruginosa* was observed to decrease, and that these changes correlated with improved lung function. [161] A reduction in *P. aeruginosa* abundance following treatment was also reported by Smith *et al.*, who noted rapid decreases in *P. aeruginosa* abundance and an associated increase in diversity following the initiation of intravenous antibiotic treatment for pulmonary exacerbations in CF, although these changes were transient and returned to baseline following the completion of treatment. [162]

1.5.9 Limitations to CF Microbiome research to date

There are a number of factors that may explain the differences between the studies described in this section and many of them apply to studies of the CF microbiome in general. The most obvious is the heterogeneous study designs, which are mostly retrospective and/or observational in nature and include a wide range of antibiotic regimens. For example, some studies included exacerbations treated with oral antibiotics as well as those requiring intravenous therapy. [150,160] Milder exacerbations are often treated with oral antibiotics and hence associated changes in the microbiota may also be expected to be more subtle. Even in those studies where only intravenous regimens were used, the antibiotic regimens or doses given are often not listed. The frequent lack of a control or comparator group further exacerbates the difficulty in interpreting results. A further consideration is the sampling timeframes in each study, where again there exists considerable variation that may have implications for interpreting results, particularly given that Smith *et al.* reported significant but transient reductions in *P. aeruginosa* abundance in the first few days of treatment. [162]

Variation also exists within and between patients in-terms of *P. aeruginosa* populations, where high levels of phenotypic diversity are typically exhibited. [68] Differing antimicrobial resistance, virulence and biofilm formation abilities could all feasibly impact on clinical outcomes of those infected with *P. aeruginosa*, however these changes would not be detected by sequencing of the 16S rRNA gene.

Given the heterogeneous nature of CF as a disease and also its associated microbiome, one strategy is to utilise a cross-over design which has been employed to good effect in a recent phase III clinical trial of a new CFTR modulator. [163] Cross-over studies allow each patient to act as their own control, minimising inter-individual variation as a confounder and improving the statistical efficiency of a study. To our knowledge only one cross-over study has been performed in the CF microbiome setting when Peleg *et al.* conducted a double-blind, placebo-controlled, cross-over study of 28 days ivacaftor treatment. [164] Sputum was collected at the start and end of each 28-day treatment period and 16S rRNA gene sequencing was performed. No significant differences were observed for either total bacterial load or *P. aeruginosa* abundance following ivacaftor therapy, and no significant difference in the microbiota composition (based on 16S rRNA microbiome analysis) was observed between the placebo and treatment samples. However, the authors noted that when they adjusted for consistent or changing antibiotic exposure within the 28-day study period, ivacaftor was associated with a significant reduction in *P. aeruginosa* abundance, that is to say, changes in the microbiota induced by acute changes in antibiotic administration during the 28-day treatment periods may have masked the effect induced by ivacaftor. It is important to note that, in clinical practice, CFTR modulators may affect the ability to collect good quality sputum samples and without confirmation of good quality samples, there are limitations as to the conclusions which can be drawn.

Bias can be introduced into sequencing results at practically any stage from sample collection, where multiple freeze-thaw cycles have been demonstrated to affect the results of microbiota analysis in respiratory samples, [298] to the sequencing itself

where different sequencing platforms can also produce different profiles. [165,166] There is no universally standardised protocol for the extraction of DNA from respiratory samples and hence methods are often inconsistent between study groups, for example the lysis of bacterial cells, which is a required initial step in order to release DNA before purification, can be performed mechanically with bead beading or enzymatically and each method can vary in their efficacy against Gram-negative and Gram-positive bacteria resulting in subsequent downstream bias. [167,168]

DNA extraction can be standardised and efficiently performed using commercial kits, however contaminating DNA is seen commonly in such kits and hence negative controls are recommended to help identify potential contamination. [169] Another striking methodological inconsistency is the use of propidium monoazide (PMA), a chemical compound that binds DNA in cells with damaged membranes and hence allows exclusion of non-viable DNA from sequencing. Excluding non-viable DNA has been suggested to be important for accurately identifying which members of the community are active at times of exacerbation, and help to avoid overestimation of viable microorganisms following treatment with antibiotics, but it is not utilised by all groups. [170,171] There are concerns that PMA may incompletely penetrate sputum, hence only identifying a portion of non-viable cells. PMA is also known to stain viable cells of some species and not stain dead cells in others. [172] In CF exacerbations, PMA treatment was not found to significantly alter the community as a whole, and only changes in low abundance “satellite” taxa were apparent. [170]

Overall, there is certainly evidence some that acute antibiotic administration alters the respiratory microbiota, however in the absence of prospective controlled trials it is difficult to interpret these results given the confounders mentioned above. Indeed, there have been calls for future clinical trials in CF to include biobanking of samples to allow more rigorous scrutiny of the effect of antibiotic agents on the microbiome. [147]

1.6 Management of infection in CF

1.6.1 Early life

The management of chronic infection in CF is targeted towards the suppression of purported pathogenic colonisers with the synchronous aim of preventing exacerbations. Strategies usually include the administration of long-term antibiotics by either inhaled and/or oral route. Some countries adopt a pre-emptive approach to chronic infection and start long-term oral prophylaxis at an early age, for example until recently UK guidelines recommended oral anti-staphylococcal prophylaxis in all infants and this strategy was historically widely adopted in the UK and Australasia. [173–175] This approach has been associated with reductions in *S. aureus* positive respiratory cultures however, a number of reviews including a Cochrane meta-analysis have found little evidence that these reductions translated into improvements in clinical outcomes and worryingly a trend towards increased *P. aeruginosa* growths has been observed. [176–178] To address this concern, a large multi-centre randomised control trial, CF START (EudraCT No: 2016-002578-11), is currently underway in the UK.

1.6.2 Eradication of *P. aeruginosa*

Given the prognostic implications of chronic *P. aeruginosa* infection, all guidelines recommend an attempt at eradication after a first *P. aeruginosa*-positive respiratory culture. [176,179–182] Nebulised antibiotics alone or in combination with oral antibiotics have been found to be superior at achieving eradication however as of yet no specific agent or regime has emerged as superior. [183] Historically, some patients were admitted to hospital for intravenous antibiotics in addition to inhaled antibiotics for targeted eradication, however the recent TORPEDO-CF study demonstrated intravenous antibiotics were not superior to oral antibiotics in terms of sustained eradication but were associated with increased treatment burden and healthcare costs.[184] Success rates of eradication vary depending on the timeframe chosen to evaluate success: At one month, eradication improves sputum-culture negativity by 90%, but by six months success rates of 30-50% are reported. [183,185,186] There is some uncertainty as to whether *P. aeruginosa* is actually

eradicated by eradication treatment, or whether its bacterial load is merely suppressed such that it cannot be detected by conventional culture methods. Supporting this, some studies have shown that *P. aeruginosa* recurrence following eradication is often by an isolate that shares an identical genotype to the isolate that triggered eradication in the first place. [187]

Once eradication has failed, focus shifts from eradication to suppression in the form of long-term nebulised antibiotics. Failure of eradication therapy to induce sputum-culture negativity is considered to represent the onset of chronic infection and failed eradication is seen more frequently in those whose baseline *P. aeruginosa* growths already had features of pathoadaptation. [188] Clinically, there are a number of criteria to define chronic infection with perhaps the most common being the Leeds criteria and the European consensus criteria (ECC). [182,189] The ECC categorise infection as chronic when three samples over 6 months with at least a month between them are positive for *P. aeruginosa*. [182] The Leeds criteria define chronic infection when >50% of cultures in the last 12 months are positive. [189] Once chronic *P. aeruginosa* infection is established it is associated with increased healthcare utilisation, morbidity and mortality in CF and hence much effort is targeted towards suppression in the form of long-term nebulised antibiotics. [190,191]

1.6.3 Anti-pseudomonal antibiotics in CF

Common antibiotic agents utilised against *P. aeruginosa* in CF include some aminoglycosides e.g. tobramycin, penicillin/beta-lactam combinations e.g. piperacillin/tazobactam, 3rd generation cephalosporins e.g. ceftazidime, monobactams e.g. aztreonam and polymyxins e.g. colistimethate. The mechanism of action and spectrum of activity for each of these classes are set out in Table 1.6.1.

In general, antibiotic choice in CF is largely empirical given results of susceptibility testing for an antibiotic do not reflect the clinical outcomes related to its use. [192–

194] This is initially counter-intuitive, but it must be kept in mind that the culture conditions during susceptibility testing are drastically unrepresentative of the CF lung. For example, the anaerobic conditions within aggregates of sputum discussed earlier are not replicated by routine aerobic bacterial culture. Susceptibility testing is also performed in a monospecies environment, yet the CF lung is a multi-species environment and inter-species interactions appear to alter susceptibility to antibiotics. [195] Furthermore, within a sputum sample or even within a colony of *P. aeruginosa*, different phenotypes co-exist and each may have different antimicrobial susceptibility. Susceptibility testing relies on selection and growth of a small number of isolates which may not represent the population as a whole.

Given some of the limitations in conventional antibiotic susceptibility testing described above, much effort has been put into deriving new methods. Different susceptibility techniques including biofilm susceptibility testing and multiple combination antibiotic susceptibility testing (also called “synergy” testing) have been developed in attempts to improve and inform antibiotic selection. However, despite these methods being designed to more closely replicate the *in-vivo* lung environment, prospective clinical trials utilising these approaches have to date, failed to demonstrate improved patient outcomes. [196–198]

1.6.4 Inhaled antibiotics and the management of chronic infection

Chronic infection with *P. aeruginosa* cannot usually be eradicated and hence treatment is targeted towards suppression of bacterial load in an attempt to prevent exacerbations and pulmonary function decline. The inhaled route offers the chance to deliver antibiotics directly to the site of infection and aerosol delivery of antibiotics has taken place since the 1940s. [199] Early attempts tended to include the aerosolisation of IV formulations, however these often contain preservatives or other constituents that can cause lung irritation. [200,201]

Table 1.6.1: Mechanism of action and coverage of commonly used anti-pseudomonal antibiotics in cystic fibrosis

Class of Drug	Mechanism of action	Spectrum of activity		
		Gram -ve	Gram +ve	Anaerobes
Aminoglycosides: e.g. Tobramycin	Inhibits bacterial protein synthesis by binding irreversibly to 30S ribosomal subunit	+	+/-	-
Penicillin/Beta-lactam e.g. Piperacillin/tazobactam	Disrupts cell wall synthesis/repair by binding to penicillin binding proteins 1b,2a,2b,3 to disrupt peptidoglycan synthesis	+	+	+
Monobactams e.g. Aztreonam	Disrupts cell wall synthesis/repair by binding to penicillin binding protein 3 to disrupt peptidoglycan synthesis	+	-	-
3rd-generation cephalosporins e.g. Ceftazidime	Disrupts cell wall synthesis/repair by binding to penicillin binding protein 1a,1b,2,3 to disrupt peptidoglycan synthesis	+	+/-	+/-
Polymyxins e.g. Colistimethate	Binds to phospholipids in the outer membrane of Gram-negative bacteria, displaces cations thereby disrupting the outer membrane allowing leakage of intracellular contents and cell death.	+	-	-
Carbapenems e.g. Meropenem	Disrupts cell wall synthesis/repair by binding to penicillin binding protein 2,3,4 to disrupt peptidoglycan synthesis	+	+	+

Aerosols deposit themselves via different processes e.g. impaction or sedimentation, dependent on their physical properties and specific preparations of antibiotic agents have been developed to optimise deposition in the lung, Table 1.6.2. Impaction occurs when large particles, travelling at speed, undergo turbulence or changes in direction, whereas sedimentation is a time-dependent deposition of particles under the influence of gravity in the context of low flow. Impaction tends to occur in the upper/central airways, whereas sedimentation is a small airways/alveolar process. Particle size also helps dictate which process occurs, with larger particles ($>8\mu\text{m}$) tending to deposit via impaction, smaller particles ($1\text{--}8\mu\text{m}$) tending to undergo gravitational sedimentation and very small particles ($<1\mu\text{m}$) may not deposit at all. [199,202,203] The main attraction to the inhaled route of antibiotic administration is the possibility for delivering large concentrations of antibiotic directly to the site of infection with substantially less systemic exposure than oral or IV administration. Pharmacokinetic studies have demonstrated that IV administration of tobramycin (10mg/kg/day) achieved maximum concentrations (C_{max}) of $29.4\mu\text{g/ml}$ and $3.9\mu\text{g/g}$ in serum and sputum respectively, whereas inhalation of 300mg tobramycin achieved corresponding C_{max} of $1\mu\text{g/ml}$ and $1200\mu\text{g/g}$ respectively. [204,205] Similarly, aztreonam lysine for inhalation (AZLI) achieved sputum C_{max} of $324\text{--}677\mu\text{g/g}$, yet serum C_{max} was $0.4\mu\text{g/ml}$, whereas IV aztreonam achieves a sputum C_{max} of $18.7\mu\text{g/ml}$ and a serum C_{max} of $228\mu\text{g/ml}$. [206–209]

The available inhaled anti-pseudomonals in the UK are presented in Table 1.6.2. Nebulised colistimethate is considered first-line in the UK and approximately 25% of people with CF are currently prescribed it. [1] Colistimethate has a relatively small evidence base but has been shown to be superior to placebo in terms of clinical symptom scores. [207] Despite use for many years in Europe, the absence of robust placebo-controlled randomised trials meant it has never been approved for use in the USA where tobramycin (TNS) is first-line. In the UK, TNS is a second-line agent with AZLI a third-line agent, and their efficacy is supported by evidence from a number of clinical trials.

The benefits of TNS were first demonstrated nearly 20 years ago in a 6 months trial of TNS (alternating 28 days on 28 days off) vs. placebo. An alternating regimen was selected based on the rationale that “drug holidays” allowed susceptible pathogens to repopulate the airways, preventing the build-up of resistance. [210,211] TNS was associated with increased lung function, reduced sputum density of *P. aeruginosa* and comparative studies against colistimethate have demonstrated superiority of TNS in terms of lung function improvement and reduced exacerbation rate. [141,212,213]

AZLI is an aerosolized lysine salt formulation of the monobactam aztreonam and has been licensed in the UK for approximately 5 years. [214] Aztreonam binds to penicillin-binding proteins of susceptible pathogens including *P. aeruginosa* and other Gram-negative pathogens. [215] IV preparations of aztreonam are formulated with arginine, which is associated with deleterious clinical outcomes when inhaled. [200] New preparations were sought for a formulation of aztreonam that could be safely used to target pulmonary infection in CF leading to development of AZLI. Evidence for AZLI use in the chronic setting comes from the two clinical trials AIR-CF1 and AIR-CF2 and their open label extension AIR-CF3 [216–218]. AIR CF1 was a placebo-controlled trial of a 28-day course of AZLI that found AZLI given three times a day was associated with improved lung function, reduced *P. aeruginosa* sputum density and improved quality of life. [216] AIR-CF2 compared an alternating regimen of AZLI and TNS to placebo and TNS over 3 months and found improved *P. aeruginosa* sputum density, quality of life and exacerbation frequency in the AZLI arm. [217] AIR-CF3 was an 18-month open-label extension which demonstrated sustained responses in lung function and quality of life. A persistent suppressive effect on *P. aeruginosa* was also seen and no increases in antibiotic resistance were reported. [218] Recently, the results of these trials were corroborated by an effectiveness study investigating the outcomes of AZLI use in a real-world population. [219] There, AZLI was found to be associated with significant improvements in lung function, weight and reductions in hospitalisation.

As well as the development of specific antibiotic preparations for inhalation, there has also been development of nebulisers designed to provide optimum delivery. Modern nebulisers such as the eFlow (Pari Medical, West Byfleet, UK) and Ineb (Profile Pharma, Zambon, Chichester, UK) utilise vibrating meshes enabling them to be much smaller than traditional nebuliser devices. The mesh is precision engineered with thousands of taper holes and as the antibiotic preparation is forced through the holes the mesh vibrates at ~116kHz to generate 2.5µm aerosolised particles. [220]

Recently, dry-powder preparations have also become available, see Table 1.6.2. These preparations have been shown to be non-inferior to nebulised treatments and are quicker and more convenient to take, however their use can be associated with increased adverse events such as cough. [221,222]

Table 1.6.2: Inhaled antibiotics currently available in the UK

Drug	Brand name	Dose	Device	
Colistimethate	Colistin*	2 million units	BD	Any
	Promixin	1 million units	BD	Ineb
	Colobreathe [#]	1.6million units	BD	Turbospin
Tobramycin	Bramitob	300mg	BD	Any
	Tymbrineb	300mg	BD	PARI LC PLUS
	TOBI	300mg	BD	Any
	TobiPod [#]	28mg	BD	Podhaler
Aztreonam	Cayston	75mg	TDS	eFlow
Levofloxacin	Quinsair	240mg	BD	eFlow

*IV preparation used for inhalation.

[#]Dry power preparation

1.6.5 Management of acute pulmonary exacerbations

As previously described acute pulmonary exacerbations are associated with considerable morbidity and mortality, however in contrast to the huge research efforts targeted towards CFTR modulators and chronic suppressive antimicrobial therapy, there is a relative lack of evidence underpinning the management of exacerbations. This is a result of a combination of factors including heterogeneity of exacerbations themselves, lack of clear definitions, gaps in our understanding of the pathophysiology and also ethical challenges in testing alternative approaches given the importance of recovery of exacerbations. Recommended practice from guidelines is often based on expert consensus and founded in practices which have been established over many years by anecdotal and observational evidence, but few prospective trials. Probably as a consequence of the lack of robust evidence there exists variation between clinicians, centres and countries in antibiotic strategies and use of adjunct therapies. [86,223] Nevertheless, goals of therapy are broadly agreed in that management is targeted towards improving lung function and relieving symptoms. [224]

When deciding whether to instigate treatment, clinicians must weigh up the constellation of symptoms, signs and lung function to establish whether there is indeed evidence of an exacerbation but also the degree of severity. Milder exacerbations, which account for over 70% of all exacerbations, can often be treated with oral antibiotics. [225] In chronic *P. aeruginosa* infection, ciprofloxacin is the first line oral agent and has been shown to be useful in this setting. [226,227] Other antibiotics such as chloramphenicol, tetracyclines and co-trimoxazole all have some activity against *P. aeruginosa* and are considered second-line. [179]

In more severe exacerbations or where there is lack of response to oral antibiotics, IV antibiotics are recommended. [173,176] IV antibiotics used commonly are presented in Table 1.6.3. Guidelines recommend the use of two IV antibiotics based on the fact that *in-vitro* synergy has been observed but also due to concerns that

resistance to monotherapy may result in treatment failure and further clinical decline. [228,229] A recent Cochrane review found combination therapy of two IV antibiotics was associated with significantly greater improvements in lung function compared to a single antibiotic approach, although overall quality of evidence supporting this approach was low. [230] The authors of the review found no evidence to support superiority of a specific antibiotic, and in practice antibiotic regimens usually consist of one antibiotic from each group set out in Table 1.6.3.

Table 1.6.3: Anti-pseudomonal IV antibiotics available for use in the UK.

Drug	Dose	Frequency
<i>Group 1: Beta-lactams/Monobactams/Carbapenems</i>		
Piperacillin/Tazobactam	4.5g	3 to 4 daily
Meropenem	1 to 2g	3 daily
Aztreonam	2g	3 daily
Ceftazidime	3g	3 daily
Ceftazidime/Avibactam	2g	3 daily
Ceftolozane/Tazobactam	1g	3 daily
<i>Group 2: Aminoglycosides/Polymyxins/Phosphonic acids</i>		
Tobramycin	3mg/kg	3 daily
Fosfomycin	4g	3 daily
Colistimethate	2 million units	3 daily
Vancomycin	1g	2 daily

People with CF have a different volume of distribution, eliminate antibiotics quicker than healthy controls and therefore often require higher doses of antibiotics. [231,232] In addition, the complex nature of infection in CF requires longer treatment durations. Typically, 10-14 days is recommended as a minimum and in the US, average duration of IV treatment is 16 days in adults and 14 days in children. [224] Longer courses can be beneficial if lung function has not returned to baseline at 14 days. [233] In addition to the high doses and extended durations of antibiotic courses used in CF, there is also evidence that the best performing CF centres are those that use the most IV antibiotics and this has been used to support an approach of treating infection early and aggressively. [234–236]

However, the benefits of IV antibiotics must be weighed against the risks. In CF, high doses and long durations result in incredibly high cumulative exposures to antibiotics and subsequently side-effects, adverse effects and long-term complications are increasingly reported. [120] Aminoglycosides in particular are nephrotoxic and ototoxic and the incidence of acute kidney injury has been reported to be as much as 100 times greater in people with CF than the general population. [237,238] It is therefore unsurprising that in the long-term, cumulative IV antibiotic courses have been inversely associated with renal function. [31] Beta-lactams do not carry the same risk of nephrotoxicity but are associated with adverse reactions ranging from mild rash to severe angioedema or anaphylaxis. Lifetime cumulative IV beta-lactam exposure is associated with an increased risk of allergic reaction which can result in severely limited antibiotic options in certain patients. [239]

IV antibiotics require venous access and hence repeated venepuncture and cannula insertions. Repeated venous access attempts can lead to increasingly difficult cannulation which is problematic for patients. Totally implantable venous access devices (TIVAD) are therefore considered in individuals requiring repeated courses of IV antibiotics or who have difficult venous access. TIVAD are more convenient for patients but serious complications including blockage, thrombosis and infection can occur. [240]

1.7 Inhaled antibiotics for the treatment of acute chest infections – A new approach?

The increasing life-expectancy of people with CF brings new challenges including increasing burden on beds/resources and also increased lifetime systemic antibiotic exposure with subsequent implications for morbidity associated to toxicity and allergic reactions. Hence, other treatment approaches must be considered and in the last five years two new inhaled antibiotics (AZLI and LIS) have become available and a number of other products including an inhaled tobramycin/fosfomycin preparation, inhaled liposomal amikacin and inhaled ciprofloxacin are in various stages of development. [241–244] Inhaled antibiotics have traditionally been reserved to suppress chronic infection, however the recent expansion of available preparations means they could now be considered for use in acute exacerbations. Many of the properties that make inhaled antibiotics useful for the treatment of chronic infection e.g. delivery of high concentration of drug to the site of infection and minimal systemic exposure also make them attractive for the treatment of acute pulmonary exacerbations. Furthermore, they may represent another treatment option for individuals with difficult venous access.

There have been concerns that areas of the lung most affected by acute infection may also have reduced ventilation, and hence inhaled antibiotics would sub-optimally deliver drugs to that area. For example, a computational fluid dynamics model predicted that in a scenario where airway liquid was thick, e.g. during an exacerbation, smaller airways would have significantly lower concentrations of AZLI than larger airways. [245] However, a recent imaging study using magnetic resonance imaging (MRI) found perfusion was also significantly decreased in areas of infection at the time of exacerbation, thus suggesting IV antibiotics may not hold superiority over inhaled antibiotics in that regard. [246]

The use of nebulised antibiotics for acute pulmonary exacerbations is not a novel idea. Nebulising IV preparations of tobramycin was reported in 1983, carbenicillin in 1985 and amikacin in 1987. [247–249] However, as mentioned previously some IV

preparations, e.g. aztreonam arginine, include lung irritants and the results of studies in the 1980s were, perhaps unsurprisingly, mixed. A Cochrane review in 2012 found only four small randomised control trials and insufficient information for meta-analyses. [250] More recently, a randomised cross-over study confirmed the benefits of TNS vs. IV tobramycin in the treatment of acute exacerbations. This was the first study to utilise a preparation optimised for inhalation and 14 days of TNS was associated with a 16% improvement in FEV1, which was similar to IV tobramycin, but TNS use resulted in renal sparing and a significantly prolonged time to next exacerbation. [251]

A recent systematic review of evidence-gaps in CF highlighted the management of acute pulmonary exacerbations as an area in need of further research. [252] Inhalation of antibiotic agents optimised for pulmonary delivery warrants further investigation and further trials are needed.

1.7.1 Inhaled antibiotics in non-CF bronchiectasis

Similarly to CF, *P. aeruginosa* is the most common pathogenic coloniser found in people with non-CF bronchiectasis (NCFB) and mortality is higher in NCFB when *P. aeruginosa* is cultured in sputum. [253,254] Three months of treatment with nebulised colistin has been reported to extend time to next exacerbation compared to placebo in people with chronic *P. aeruginosa* infection and inhaled ciprofloxacin has had some success, although recent phase 3 clinical trials were conflicting. [243,255,256] Long-term inhaled aztreonam was not found to be beneficial with NCFB, although post-hoc analysis revealed clinical improvements were seen in the sub-group with high sputum bacterial load at baseline. [257,258] Other evaluated agents include gentamicin, where in a study of 65 adults with NCFB, 12 months of nebulised gentamicin reduced exacerbation rate compared to placebo in people with *H. influenza* or *P. aeruginosa* infection. Recent guidelines therefore recommend nebulised antibiotics for people with chronic *P. aeruginosa* infection in NCFB. [259–261]

In the acute setting, relatively little is known about the feasibility of inhaled antibiotics for treatment of exacerbations of NCFB as only two studies have been conducted. The first was a multi-centre study comparing the addition of nebulised tobramycin or placebo to oral ciprofloxacin for treatment of exacerbations in people with NCFB and *P. aeruginosa*. [262] The study recruited 53 patients and although there was significantly greater microbiological efficacy seen in the nebulised arm, no corresponding clinical improvements were noted. More recently, a Chinese study explored the efficacy and safety of the addition of nebulised amikacin to standard IV antibiotic therapy for treatment of exacerbations in adults with NCFB and reported similar clinical outcomes between groups, but significantly greater sputum bacterial eradication rates with nebulised amikacin. [263] In both studies, nebulised antibiotics were tolerated well, although wheeze (a recognised side-effect of nebulised tobramycin preparations) was reported to be higher in those receiving tobramycin in the earlier study.

1.7.2 Inhaled antibiotics in ventilator-associated pneumonia

Ventilator-associated pneumonia (VAP) occurs in 10-27% of intubated critically ill patients and accounts for up to 50% of antibiotic use in intensive care units. [264] The use of aerosolised antibiotics has been advocated in this population given the high rates of multi-drug resistant pathogens (including *P. aeruginosa*) and the theoretical ability to deliver high concentrations of the drug to the site of action. A number of different antibiotic agents, preparations and delivery devices have been investigated including those previously studied in CF e.g. colistimethate, tobramycin, and amikacin. [265–267] Additionally, novel preparations e.g. an amikacin-fosfomycin combination nebulised therapy have also been studied. [268] Results from these studies are mixed, probably reflecting the wide range of preparations used and challenges delivering aerosols to mechanically ventilated patients. [269] Nevertheless, in studies with preparations and delivery optimised for intubated patients, positive effects on bacterial load, antibiotic resistance, time to intubation and other signs and symptoms of infection have been reported. [270,271]

One systematic review and meta-analysis of a heterogeneous range of study designs, interventions and comparators found nebulised antibiotics were associated with improved short-term clinical outcomes but no microbiological cure or mortality benefit. [266] A further review only considered the role of colistimethate and found improved clinical response, microbiological eradication and reduced infection-related mortality when nebulised colistimethate was added to standard IV therapy. [271] Subsequently, in 2016 the American Thoracic Society recommended the use of nebulised antibiotics for VAP in certain circumstances, namely when the causative organism was a Gram-negative bacilli exhibiting some degree of antimicrobial resistance. [266,272] More recently, a European meta-analysis found evidence of improved clinical cure rate and reduced mechanical ventilation duration but no effect on overall mortality for nebulised antibiotics used as either an adjunct to, or substitute for, IV antibiotics. [273] Nephrotoxicity was reduced when nebulised antibiotics were used, but there was a 9% increase in respiratory complications, which appeared to particularly affect those with severe acute respiratory distress syndrome, and a European consensus statement therefore suggested more research was required before nebulised antibiotics could be routinely recommended . [273,274]

1.7.3 Inhaled antibiotics in chronic obstructive pulmonary disease

To date, only gentamicin has been evaluated in the acute COPD exacerbation setting, although nebulised tobramycin was associated with reduced sputum inflammation in stable COPD patients. [275,276] The gentamicin study was an Iranian double-blinded placebo-controlled clinical trial in hospitalised patients with an acute exacerbation of COPD. 86 participants were randomly assigned to the addition of nebulised gentamicin or placebo (0.9% sodium chloride) to oral antibiotics for five days. Both treatments improved dyspnoea scores and the nebulised gentamicin group saw greater improvement in lung function. [276]

1.7.4 Current use of inhaled antibiotics for acute pulmonary exacerbations in CF

Observational studies report 10-24% of patients treated for an exacerbation received inhaled antibiotics; however it is not clear from these studies whether these were short courses for exacerbation treatment or the continuation of long-term suppression. [223–225,277] Given the lack of evidence for or against their use in this setting there have been calls for further studies to address the evidence gap for the approach. [223,278]

1.8 Conclusion

CF pulmonary disease is characterised by progressive chronic infection, punctuated by periods of acute deterioration termed exacerbations. Exacerbations are associated with significant morbidity and increased mortality and currently require aggressive management in the form of two IV antibiotics for extended periods. Such treatment can be associated with complications, side-effects and increased antimicrobial resistance, therefore new strategies are required. Inhaled antibiotics, a well-established weapon in the management of chronic CF lung infection, may represent a new treatment strategy in the management of acute infections and warrant further investigation.

1.9 Aim of this thesis

- Aim 1:** Establish the current usage of inhaled antibiotics in the acute setting amongst CF clinicians in the UK.
- Aim 2:** To investigate the clinical utility of AZLI plus IV colistin against dual IV antibiotics for the treatment of acute pulmonary exacerbations of cystic fibrosis.
- Aim 3:** To compare the microbiological effect of each treatment

Chapter 2: What is Current Practice? A Nationwide Survey of the Use of Inhaled Antibiotics for Acute Pulmonary Exacerbations of CF

2.1 Introduction

The recent expansion of available preparations of inhaled antibiotics has led to the consideration of using inhaled antibiotics in place of intravenous antibiotics for some pulmonary exacerbations. [279] Inhaled antibiotics may hold benefits over intravenous antibiotics given they deliver a higher dose directly to the lung, with associated reduced systemic exposure (with potential for reduced allergies, intolerance and off target sequelae) as well as reduced need for intravenous access. In the USA, observational studies report 10-24% of patients treated for an exacerbation received inhaled antibiotics yet it is not clear whether these were new acute treatment courses or continuation of long-term suppression. [223–225] The most recent clinical trial in this area was conducted in the UK and there is anecdotal reporting of their use. [251,280] However, UK guidelines make no comment regarding the use of inhaled antibiotics in the acute setting and the extent to which inhaled antibiotics are being employed in the acute setting in CF in the UK is unknown. The aim of this study was therefore to explore, understand and benchmark usage of inhaled antibiotics for the treatment of acute pulmonary exacerbations of CF in the UK.

2.2 Methods:

An online platform (SurveyMonkey Inc, USA) was used to create a survey for national distribution amongst clinicians involved in the care of people with CF. The survey consisted of three main sections; 'Demographics', 'Usage' and 'Barriers'. These sections aimed to establish firstly which CF centre and in which setting (adult or paediatric) the respondent worked. Secondly, information regarding their use of inhaled antibiotics in the acute setting and the final section sought to elucidate perceived barriers to current and future use. The survey consisted of 9 questions

(Table 2.2.1), and was circulated via the UK Cystic Fibrosis Medical Association (UKCFMA) and also the UK CF Network. The UKCFMA is made up of physicians with specialist interest in CF from both the adult and paediatric setting. The UK CF Network is an online forum for professional discussion between UK CF clinicians. The UK has 28 adult CF centres (16 with greater than 200 patients) and 31 paediatric CF centres (6 greater than 200 patients). [15] The survey was open between February and March 2019. In addition to analysis of overall trends, subgroup analyses were performed based on adult/paediatric setting, size of centre and prior training of the respondent. Differences were compared using Fisher's exact test or Mann-Whitney's test for categorical and continuous data respectively. All statistical analyses were conducted in RStudio (v1.0.136, R Studio Inc). A p -value of <0.05 was considered significant and unadjusted p -values are presented throughout.

Table 2.2.1: Description of questionnaire sent out to CF clinicians across the UK

Question	Details
Which CF centre do you work at?	<i>Open question</i>
Adults or paediatric?	<i>Open question</i>
How many patients at your centre?	<i>Open question</i>
Have you undertaken a period of CF training abroad?	<i>Yes/No</i>
Have you used inhaled antibiotics in place of intravenous antibiotics for the treatment of an acute pulmonary exacerbation of CF?	<i>Multiple choice: Never/Rarely/Often</i>
Which inhaled antibiotics have you used?	<i>Tickbox from a list of antibiotics</i>
What is your overall experience in using inhaled antibiotics for the treatment of acute pulmonary exacerbations in CF?	<i>Five-point Likert scale</i>
What do you feel are the main barriers to increased use of inhaled antibiotics in the acute setting?	<i>Tickbox from a list of possible barriers with freehand option</i>
How do you foresee your use of inhaled antibiotics in the acute setting will change in future?	<i>Multiple choice: Increase/Stay the same/Reduce</i>

2.3 Results:

The survey was accessed by 35 respondents and completed by 34 (97% completion rate). Respondents represented 25 different CF specialist centres across the United Kingdom (see Figure 2.3.1) providing care for 6850 people with CF (two-thirds of the UK CF population). [1] Overall, 21 (62%) respondents were primarily involved in the care of adults with CF, representing 15/28 (53.6%) of all adult specialist CF centres in the UK. 13 respondents were primarily involved in the care of children with CF, representing 13/31 (42%) of all paediatric specialist CF centres.

2.3.1 Use of inhaled antibiotics

Twenty-five respondents (74%) reported previous use of inhaled antibiotics to treat acute pulmonary exacerbations of cystic fibrosis and there was no significant difference in practice between adult or paediatric clinicians (17/21, 81% vs. 8/13, 62% respectively, $p=0.25$), see Figure 2.3.2. Four respondents reported a period of CF training abroad and all four (100%) reported the use of inhaled antibiotics in the acute setting compared to 21/30 (70.0%) of those with no period of training abroad, however this numerical difference was not statistically significant ($p=0.29$). As regards frequency, only 3 /25 (12.0%) reported use often.

Overall experience was evaluated using a 5-point Likert scale. Only 2/25 (8%) respondents reported their experience using inhaled antibiotics was negative with the majority describing neutral experience and 10/25 (40%) describing positive or very positive experience, see Figure 3. Where usage was reported, colistimethate, tobramycin and aztreonam were the most frequently reported antibiotics used, see Figure 2.3.4.

Figure 2.3.1: Map of the United Kingdom showing distribution of respondents



Figure 2.3.2: Percentage of respondents reporting any use of inhaled antibiotics in the treatment of acute pulmonary exacerbations of CF

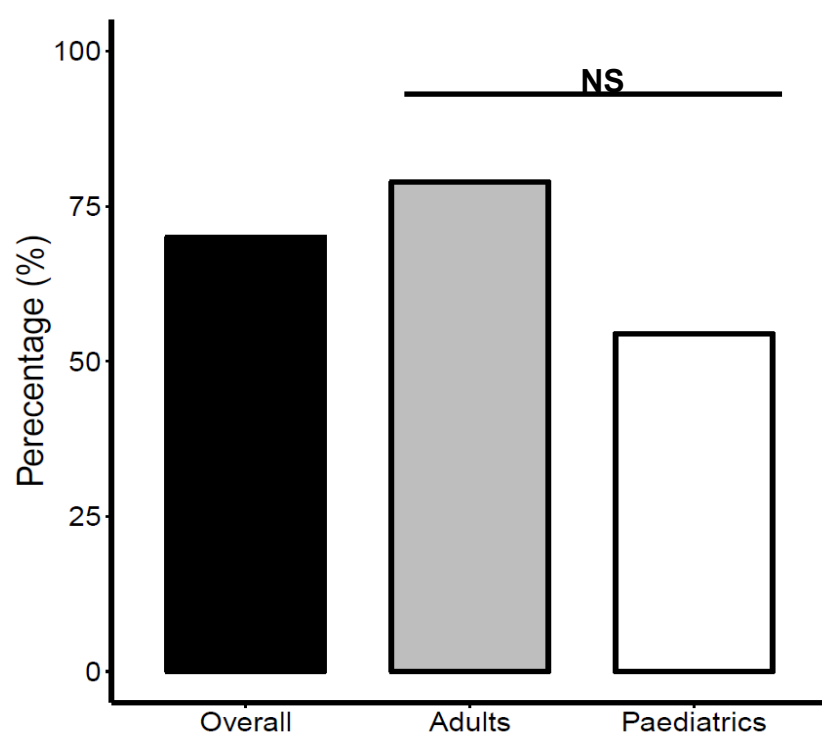


Figure 2.3.3: Likert scale responses to the question “What is your overall experience in using inhaled antibiotics for the treatment of acute pulmonary exacerbations in CF?”

(1=Very Negative, 2=Negative, 3=Neutral, 4=Positive, 5=Very Positive)

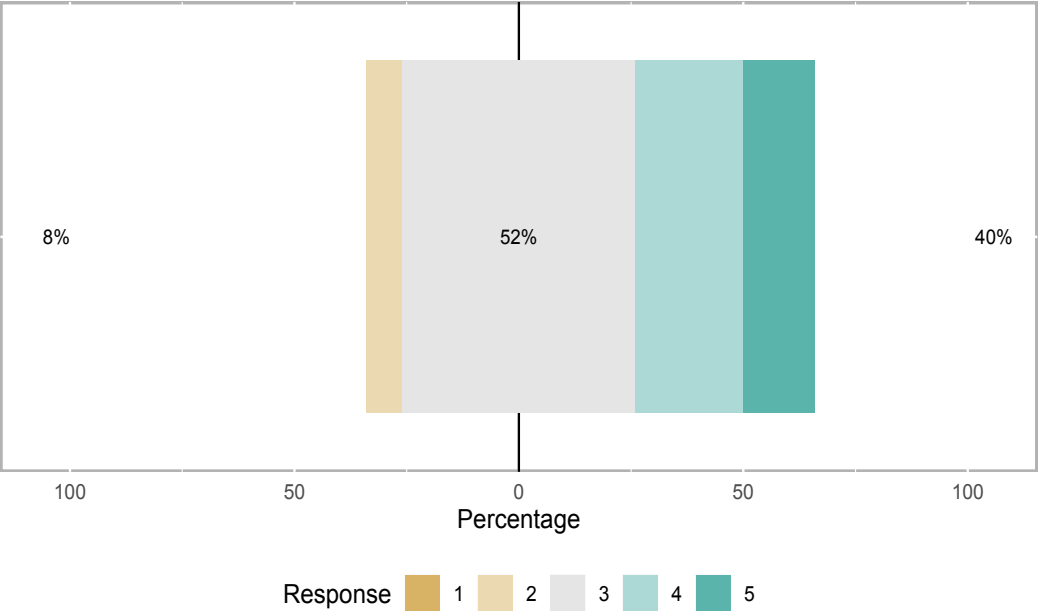
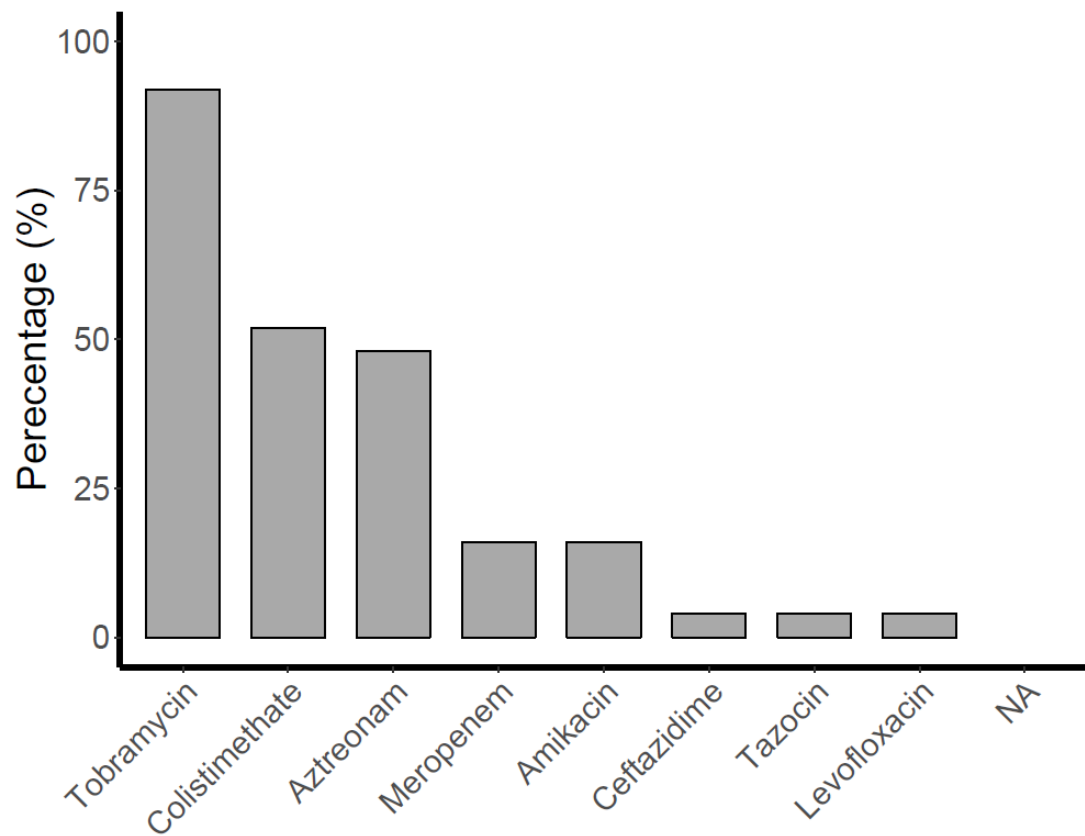


Figure 2.3.4: Percentage of respondents (n=24) with prior use of inhaled antibiotics in the acute setting who reported previous experience for each antibiotic.



2.3.2 Perceived barriers

The next section explored respondents' perceptions of potential barriers against the use of inhaled antibiotics in the acute setting. "Inadequate delivery of drug to the site of action" was identified by 14/33 (42%) of respondents and was the most frequently identified barrier. Other frequently identified barriers included "side-effects e.g. coughing": 9/33 (27%), unlikely clinical benefit: 10/33 (30%) and patient choice 6/33 (18.2%), see Table 2.3.1

2.3.3 Future use

Finally, respondents were asked how they expected their use of inhaled antibiotics would change in the next few years. No respondents expected use to decline. All clinicians reporting they had never used inhaled antibiotics in the acute setting expected their practice to remain unchanged. Conversely, those who had used inhaled antibiotics were significantly more likely to increase their use in the next few years (12/25 prior users reported they were likely to increase use compared to 0/9 non-users, $p < 0.01$). No users felt their use would reduce in the future.

Table 2.3.1: Perceived barriers to the use of inhaled antibiotics in the treatment of acute pulmonary exacerbations of CF.

Potential Barrier	No. respondents	%
Inadequate delivery to the site of action, e.g. reduced ventilation in area of acute infection	14	42.4
Unlikely clinical benefit	10	30.3
Side-effects, e.g. coughing	9	27.3
Patient choice	6	18.2
Limited available agents/preparations	5	15.2
Cost	3	9.1
Selected others (all n=1)		
<i>"Lack of evidence base"</i>		
<i>"Technicalities of giving these drugs on a ward, storage in pts rooms, fridge size. Drug interactions."</i>		
<i>"Difficult to do in acute setting with physio provision"</i>		
<i>"Unlikely to aide systemic effects of infection"</i>		
<i>"Good in mild exacerbations not proven in more severe and patients can be too unwell to tolerate them"</i>		

2.4 Discussion

This study explored the current nationwide usage of inhaled antibiotics for the treatment of acute pulmonary exacerbations of CF. Our findings demonstrate that the majority of CF centres have used inhaled antibiotics in the acute setting, however there is variation in practice and only a minority of centres/clinicians routinely use this approach.

In the US, guidelines for the management of young children with CF recommend the use of inhaled antibiotics as part of the management of pulmonary exacerbations. [279] However, we found no significant difference between usage in the adult and paediatric settings in the UK and if anything, a trend towards less use in paediatrics. These findings may be explained by the challenges in administering nebulisers to young children, who may not be able to follow instructions adequately to achieve optimal delivery of the antibiotic into the lung. Furthermore, most antibiotics optimised for inhalation are targeted towards *P. aeruginosa* which is found in fewer children than adults and hence it is unsurprising that paediatric use of inhaled antibiotics remains low.

Data from previously published studies suggest that in healthcare settings outside the UK, the rates of use of inhaled antibiotics during acute pulmonary exacerbations may be as high as 24%. [225] Accordingly, all three clinicians (from three centres, one paediatric and two adults CF clinicians) who had completed a period of CF training outside the UK had used inhaled antibiotics in the acute settings, compared to two-thirds of those trained exclusively in the UK.

Although UK guidelines make no recommendations for or against the use of inhaled antibiotics in the acute setting, the most recent study in this area was conducted in the UK and found substituting nebulised tobramycin for intravenous tobramycin was effective in treating exacerbations and was superior in terms of time to next exacerbation. [251] As such it is perhaps of no surprise that tobramycin was the most frequently reported inhaled antibiotic agent used by respondents to this study.

This study explored perceived barriers/concerns for the use of inhaled antibiotics in the acute setting and the most frequent concern related to inadequate delivery of drug to the site of action. This was an unexpected finding given that inhaled antibiotics deliver a significantly greater dose of antibiotic to the lung. One interpretation of this finding is that clinicians may perceive that although a greater dose of antibiotic is delivered to the lung itself, the site of infection may be plugged with excess mucus and therefore not ventilated adequately. The aerosolised drug would instead be deposited in higher concentrations in areas of increased ventilation, i.e. healthy lung, and hence concentrations at the site of infection may be sub-inhibitory. In such scenarios intravenous antibiotic delivery may be perceived as superior, however a recent study using MRI perfusion imaging found areas of airway obstruction are associated with local pulmonary vasoconstriction and subsequent perfusion deficits. [246] This mechanism is a physiological compensatory response to reduced ventilation and helps direct blood to areas better ventilated areas of the lung. Thus, intravenous administration may not actually hold superiority in terms of delivery of drugs to the site of infection.

Another interpretation of clinicians' perception that inhaled antibiotics are inadequate in terms of delivery to the site of action is they feel that some degree of systemic antibiotic exposure is necessary for optimal treatment of acute pulmonary exacerbations. The relationship between systemic inflammation and pulmonary exacerbations in CF is complex, for example C-reactive protein (CRP), does not appear to be related to exacerbation severity, but a very elevated admission CRP is highly specific for a poor treatment response. [281,282] Clinicians may therefore have fears that the reduced systemic exposure of inhaled antibiotics may not sufficiently treat the systemic component of an acute pulmonary exacerbation of CF. Downey *et al.* demonstrated IV antibiotic treatment of pulmonary exacerbations was associated with reduced circulating CD11b stained neutrophils, a marker of systemic inflammation. [283] However, the same study found IV antibiotics were not associated with any reductions in airway inflammation, and it is not known whether

modulating the local inflammatory process in the lungs would alter exacerbation outcomes.

Our findings suggest that the use of inhaled antibiotics in the acute setting will increase in the coming years given no respondents felt their use would decrease and over a third felt their use would increase. The lack of evidence in this area was recently highlighted in two systematic reviews and since use is likely to increase, studies are needed to allow optimisation and standardisation of practice. [252,284]

The limited availability of antibiotic agents optimised for inhalation has historically been a barrier to the use of nebulised antibiotics in the acute setting, available preparations being used predominantly for chronic suppression of *P. aeruginosa*. The use of various intravenous preparations via the inhaled route has been tested in the past, but this can be associated with lung irritation and is therefore inappropriate during exacerbations. [200,249] Although in our study some clinicians highlighted that the lack of available preparations still remains a barrier today, the number of licensed agents has increased in the last ten years. A number of other agents are also undergoing clinical trials, e.g. fosfomycin/tobramycin combination, liposomal amikacin, and dry-powder ciprofloxacin, all in the non-acute setting and predominantly targeted towards chronic suppression of *P. aeruginosa* in a clinically stable population. [242–244] Repurposing of these drugs towards the acute setting could potentially yield an increase in treatment options for acute pulmonary exacerbations of CF and further studies are needed.

Limitations to this study lie mainly in recall bias associated with all studies of this kind and the fact that responses were not received from all CF centres in the UK. The survey was distributed amongst the UKCFMA and UK CF Network and a potential selection bias could be that clinicians engaged in these forums are more likely to adopt or try new practices and the use of inhaled antibiotics in the acute setting would therefore be overestimated. Additionally, practice may vary within centres, between clinicians and our results may therefore not be entirely generalisable. We

did not ask respondents whether they had used inhaled antibiotics as a primary treatment strategy or as secondary/salvage therapy once other treatments had failed and are therefore unable to comment on inhaled antibiotic utility in those distinct settings. Nevertheless, the strengths of the study lie in the coverage of over two-thirds of the UK CF population and over 70% of adult CF centres in the UK. This is the only study, to our knowledge, exploring current practices and perceptions regarding the use of inhaled antibiotics for acute pulmonary exacerbations and can inform future research directions in order to standardise and optimise this treatment strategy.

In conclusion, the majority of CF clinicians in the UK have used inhaled antibiotics to treat acute pulmonary exacerbations. Very few reported negative outcomes and usage appears set to increase in the coming years. Research defining the appropriate agents and regimens is required.

Chapter 3: AZTEC-CF Methods and Study Design

3.1 Introduction

AZTEC-CF (Aztreonam lysine for inhalation for the treatment of exacerbations of cystic fibrosis) is an open-label, pilot single centre (Liverpool Heart & Chest Hospital NHS Foundation Trust), randomised cross-over pilot trial designed to investigate the efficacy of AZLI in the treatment of acute pulmonary exacerbation of CF. Study documents are deposited on the github.com repository available at <https://github.com/Ffrost2/AZTEC-CF>.

3.1.1 Study rationale

Inhaled antibiotics deliver an extremely high concentration of drug to the lung, which can be in the order of 300 times greater than IV antibiotics, with significantly less systemic exposure. With this in mind, using inhaled antibiotics in the acute setting may treat acute lung infection more effectively while also reducing some of the complications associated with repeated prolonged systemic antibiotic exposure. The recent expansion of available preparations optimised for inhalation means there are now more options available for consideration of use in the acute setting, however, there are concerns inhaled antibiotics may not treat the systemic component of exacerbations or may not reach the area of infection due to reduced ventilation. This study aims to further our understanding of the efficacy, safety and microbiological implications of using inhaled antibiotics to treat acute exacerbations.

3.1.2 How does this study meet the research needs of the CF community?

A recent priority setting partnership, facilitated by the James Lind Alliance, helped define the top 10 list of priority clinical research questions in CF. [285] The process included elicitation and prioritisation surveys of patient and clinical communities. Investigating the use of inhaled antibiotics in the acute setting addresses three of the research priorities set out by the JLA process. Additionally, a recent systematic review of areas which lack evidence for treatment decisions in CF highlighted a gap in the evidence for exacerbation management and determining most effective route of antibiotics administration during exacerbation. [252] These findings are further

supported by the recent Cochrane review of inhaled antibiotics for the treatment of acute exacerbations which concluded more research is needed in this area. [284]

3.2 Study objectives

Primary objective

- To investigate whether there is immediate clinical benefit in the use of AZLI in acute exacerbations of CF.

Secondary objectives

- To investigate what effects AZLI+IV has on lung microbiology compared to IV+IV

3.3 Study design

3.3.1 AZTEC-CF design

AZTEC-CF was a single centre pilot study at Liverpool Heart and Chest Hospital NHS Foundation Trust (LHCH). LHCH is a regional CF centre in the north-west of England, the fifth largest centre in the country, delivering care to over 300 adults with CF. The study design was a randomised cross-over study comparing AZLI plus IV colistimethate (AZLI+IV) against standard care of two intravenous antibiotics (IV+IV). Each participant received both treatments in a random sequence across two separate acute exacerbations. AZLI+IV rather than dual inhaled antimicrobials was chosen given concerns the inhaled route alone may not treat any potential systemic component of an acute pulmonary exacerbation. Colistimethate was selected over tobramycin for a number of reasons. Firstly, colistimethate remains first-line treatment in the LES cohort at LHCH. The prevalence of LES is high at LHCH and overall colistimethate resistance remains low in this population. [286] Secondly, UK guidelines recommend a standardised dosing regimen for colistimethate of 2 MU TDS in adults with CF. [173] This negates the need for extra venepuncture and dose adjustments as compared to Tobramycin. It was felt by the study team that, given the small nature of the trial, any potential under dosing and subsequent requirement

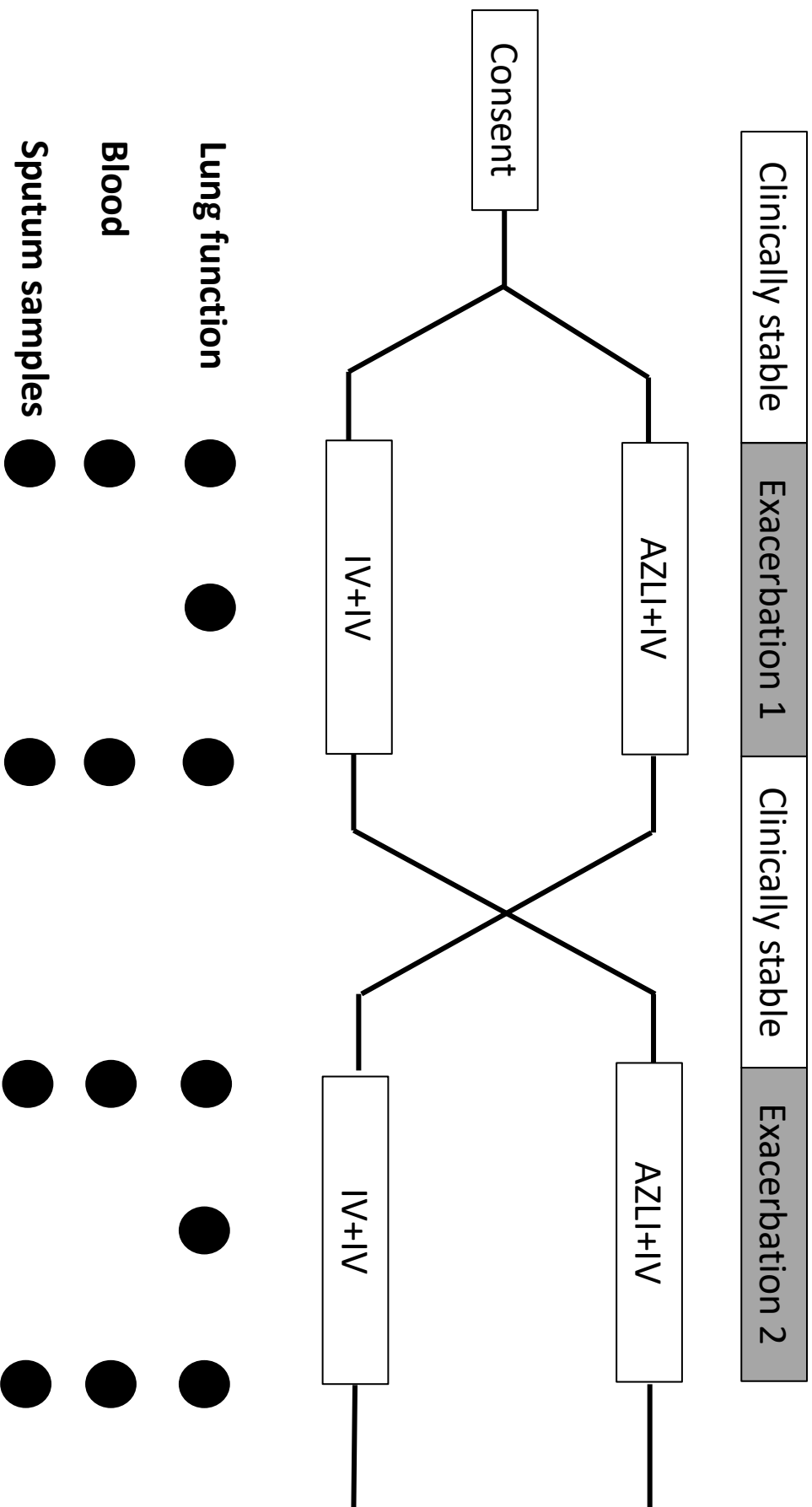
for prolonged courses would introduce an intolerable new risk of bias into study results and colistimethate was therefore considered preferential. Finally, the impact of each agent on lung microbiology was considered given the stated aims of the study. In order to be able to draw conclusions about the effects of AZLI+IV against IV+IV it was important to have some understanding of the likely effect of the IV agent used in both arms. Neither colistimethate nor tobramycin has been particularly well characterised in terms of their effects on the lung microbiota but colistimethate use has been rigorously tested in a sheep CF model. [287] Colistimethate was again felt to hold superiority over tobramycin in that regard and on those bases, it was included as the IV agent in both arms.

The cross-over design, see Figure 3.3.1, was chosen in an attempt to mitigate some of the heterogeneity of CF exacerbations. Cross-over designs are not suitable in all circumstances but have been suggested to be particularly useful in evaluating interventions with a temporary effect in chronic but symptomatic conditions and it was therefore felt to be appropriate to apply this design to the treatment of pulmonary exacerbations of CF. [288,289] Furthermore, cross-over studies improve the statistical power of a study thereby requiring smaller sample sizes and improving the efficiency of the study.

3.3.2 Funding

AZTEC-CF was funded by an investigator supported research grant from Gilead Sciences (IN-UK-205-4065). The funder had no role in the study conduct, data analyses or manuscript preparation.

Figure 3.3.3.1: AZTEC-CF study scheme



3.3.3 Eligibility criteria

Inclusion criteria

1. Confirmed diagnosis of CF
2. Patients aged 16 - 65 years of age who can give informed consent
3. FEV₁ >25% or <75% predicted
4. Receive inpatient treatment for acute pulmonary exacerbations of CF at LHCH
5. Presence of *P. aeruginosa* in lower respiratory tract cultures in the 6 months prior to screening

Exclusion criteria

1. Documented allergy to beta-lactam antibiotics or IV colistimethate
2. Growth of *Burkholderia cepacia* Complex (BCC) within 1 year
3. Pregnancy
4. Previous organ transplant
5. Receiving other clinical trial medication
6. Already prescribed regular AZLI
7. Hypersensitivity reaction to AZLI excipient
8. Hypersensitivity reaction to polymyxin B
9. Patients with myasthenia gravis
10. Any contraindication to the use of the chosen standard intravenous antibiotic.

3.3.4 Study approvals

Local approvals

The study was approved locally by the Liverpool Heart and Chest Hospital Research and Innovation Committee following internal review and discussion at committee meeting (2016AZLIDN001/1124). The study concept, protocol and documents were also discussed and approved by the local patient group, the SURE group.

Regulatory approvals

Ethical approval was provided by the North West -Haydock Research Ethics Committee (16/NW/0741) in November 2016. MHRA clinical trial authorisation (CTA) was also received in November 2016 (21422/0007/001-0001). Health Research Authority approval was obtained in December 2016. The study was registered with EudraCT (2016-002832-34) and ClinicalTrials.org (NCT02894684).

3.4 Participant identification, recruitment and randomisation

Participants were recruited from the outpatient clinic at the adult CF centre at Liverpool Heart and Chest Hospital NHS Foundation Trust. All potential participants were identified from the local registry using lung function and microbiology eligibility criteria. This approach identified 65 individuals and recruitment was by one of two approaches. Firstly, a patient information sheet (PIS) was posted to all potential participants individually to inform them of the study. Potential participants were asked, if interested, to contact a member of the research team. The second approach to recruitment was to approach potential participants directly in the outpatient department. In practice participants were recruited from both approaches, with 6/1 recruited directly via the first approach and 10/16 via the second. More details are provided in Figure 4.3.1.

3.4.1 Consent

All participants interested in enrolling were seen by a member of the study team who explained the study in more detail, confirmed inclusion and exclusion criteria and obtained fully informed written consent. In practice this was performed in an

outpatient clinic appointment. Participants were only randomised to treatment at the time of their first admission, see 3.4.4.

3.4.2 Baseline investigations

At the time of consent, all patients received a unique identifier and baseline clinical characteristics (age, sex, genotype, lung function, body mass index, recent sputum microbiology, annual intravenous antibiotic days), past medical history and medication history were all recorded.

3.4.3 AZLI test dose

A test dose of AZLI was administered by a CF specialist physiotherapist in order to assess for side-effects or evidence of an exaggerated bronchoconstriction response. Severe wheeze or a post-dose drop in FEV1 of 10% was considered an exclusion to continuation within the study.

3.4.4 Randomisation

Block randomisation in groups of four was carried out prospectively using Statsdirect® (Statsdirect Ltd. 2013). A randomisation scheme was generated indicating the treatment arm each participant would initiate at the point of their first exacerbation. This was performed prospectively, and the randomisation scheme was referred to only at the point of randomisation. Point of randomisation was the point at which participants were admitted to Liverpool Heart and Chest Hospital for treatment of a protocol defined pulmonary exacerbation.

3.4.5 Blinding

Neither study investigators nor participants were blinded to the treatment during each admission. The different modalities of the respective interventions meant blinding would have required dummy or sham nebulisations and infusions. The logistical impediments this would have on the day to day running of the study were felt to make them practically infeasible. In an effort to minimise bias, outcomes were selected such that all measurements were objective and either measured or overseen by an individual blinded to treatment allocation.

3.4.6 Definition of pulmonary exacerbation

A pulmonary exacerbation was defined as per the modified Fuch's criteria set out by the EuroCareCF working group in 2011:

Two or more of the following:

- Change in sputum volume or colour
- Increased cough
- Increased malaise, fatigue or lethargy
- Anorexia or weight loss
- Decrease in predicted FEV1 or 10% or more
- Increased dyspnoea. [88]

3.5 Study procedures

3.5.1 Study interventions

Over the course of two exacerbations, participants sequentially received 14 days of the two treatments detailed below. For IV+IV, antibiotic selection was at the discretion of the admitting clinician.

Treatment A (AZLI+IV):

AZLI 75mg TDS via nebuliser
PLUS
Colistimethate 2MU TDS intravenously

Treatment B (IV+IV):

Colistimethate 2MU TDS intravenously
PLUS ONE OF:
Meropenem 1g TDS intravenously,
Piperacillin/tazobactam 4.5g TDS intravenously,
Ceftazidime 3g TDS intravenously
Aztreonam 2g TDS intravenously
Fosfomycin 4g TDS intravenously

A research prescription was supplied by the hospital pharmacy and included 14 days of stock for all components of the respective treatment. AZLI was administered via an eFlow® rapid nebuliser system (PARI Respiratory, Germany). The eFlow® device is an electronic nebuliser which generates aerosols as liquid passes through a vibrating stainless-steel membrane. All late phase clinical trials of AZLI have used the eFlow® device and AZLI is therefore only licensed for use via this device. Instruction on the use of the device was provided to participants by specialist CF physiotherapist.

3.5.1.1 Completion of 14 days treatment

AZLI+IV and IV+IV were both continued for 14 days at which point participants could be discharged home, as long as the clinical team were satisfied with resolution of pulmonary exacerbation. If after 14 days of treatment the clinical team felt that the pulmonary exacerbation was not suitably resolved, then participants were switched to regular stock antimicrobial therapy consisting of two intravenous antibiotics selected in line with local formulary by the clinical team. The requirement for more than 14 days of antibiotics was recorded as an adverse event.

3.5.1.2 Early termination of treatment

If the clinical team felt at any point that the allocated study treatment was failing to sufficiently improve clinical status and continuing was no longer in a participant's best interests, then with the agreement of the participant, study treatments were discontinued and standard antimicrobial therapy selected from local formulary was commenced. Participants continued in the study and all routine assessments were performed as normal at day 7 and day 14 but early terminations of treatment was recorded by the study team and if associated with clear clinical deterioration the relevant adverse event reporting forms were completed.

3.5.1.3 Concomitant medications and adjunctive therapies

In order to ensure the study was pragmatically integrated with standard clinical care, there were no restrictions on concomitant medications or adjunctive therapies. Initiation and/or changes in both concomitant medications and adjunctive therapies were recorded at day 7 and day 14.

3.5.2 Study Assessments

Study assessments were conducted on day 1, 7 & 14 as set out in the schedule of events below. A tolerance of +/- 1 day was allowed.

3.5.2.1 Day 1

Participants were seen by a member of the study team who confirmed ongoing consent to participation and any remaining questions were answered. Spirometry (if not performed in the previous 24 hours) was performed and blood was collected for routine haematology and biochemistry. A quality of life questionnaire was completed by the participant and a fresh expectorated sputum sample was collected.

3.5.2.2 Day 7

On day 7, spirometry was performed, and participants were seen by a member of the study team who assessed any adverse events and recorded any changes in concomitant medications. Blood was collected for routine haematology and biochemistry.

3.5.2.3 Day 14

On day 14, the final day of treatment, participants were again seen by a member of the study team. Adverse events and changes in concomitant medications were recorded. Blood was collected for routine haematology and biochemistry. Spirometry was performed, a quality of life questionnaire was completed, and expectorated sputum collected. Participants were thanked for their on-going participation.

3.5.2.4 Study completion

Participants were considered to have completed the study at the point of their third admission with acute pulmonary exacerbation, such that days to next exacerbation from the second admission were recorded.

3.5.2.5 Spirometry

Spirometry was performed prior to study drug initiation on day 1, and then in the morning on day 7 and 14 of admission. If a participant had performed spirometry as part of an outpatient clinic <24 hours prior to admission, then this was deemed acceptable in lieu of a repeat day 1 spirometry. All spirometry was performed in the pulmonary function laboratory at Liverpool Heart & Chest Hospital by an accredited respiratory physiologist blinded to treatment allocation. Spirometry was performed in Spirostik™ (Geratherm Respiratory GmbH, Germany). Of primary interest was the forced expiratory volume in 1 second (FEV1), as percentage of predicted values. All predicted reference ranges were calculated using the Global Lung Initiative reference equations. [290]

3.5.2.6 Blood sample collection

Blood was collected on day 1, 7 and 14 in S-Monovette® blood collection tubes (Sarstedt, Germany), and sent for routine processing in the local accredited NHS clinical laboratory.

Table 3.5.1: Schedule of assessments for AZTEC-CF Study

	Day 1	Day 7	Day 14
Confirm consent	X	X	X
Medication history	X	X	X
Physical examination	X	X	X
Bloods	X	X	X
Spirometry	X	X	X
CFQ-R	X		X
Sputum sample	X		X
Assess for Adverse Events	X	X	X
Record adjunctive medication/therapies	X	X	X

3.5.2.7 Health related quality of life

Participants were asked to complete a CFQ-R questionnaire on day 1 and day 14. The CFQ-R is a validated disease specific health-related quality of life (HRQoL) tool. The questionnaire addresses a number of generic and disease specific domains including 'Physical', 'Vitality', 'Emotion', 'Eating', 'Treatment burden', 'Health Perceptions', 'Social', 'Body Image', 'Role', 'Weight', 'Respiratory' and 'Digest'. The CFQ-R consists of 50 scaled questions which are weighted to give a score on a 0-100 scale for each domain, where a higher score indicates better HRQoL. CFQ-R domain scores were generated by the data input tool provided by the questionnaire developers (<https://cfqr.github.io>).

3.5.2.8 Sputum collection

Sputum was collected on day 1 and day 14. Participants were asked to expectorate into a sterile specimen collection pot which was immediately sealed and placed in a 4°C fridge. Within 24 hours, samples were subsequently separated into three 2ml aliquots and flash frozen in liquid nitrogen prior to storage at -80°C.

3.5.3 Concomitant medications and adjunctive therapies

No restrictions were placed on participants with regards to concomitant medications or adjunctive therapies. All non-IMP related management decisions were left to the medical team looking after each participant. The use of concomitant medication and adjunctive therapies was recorded for comparisons between groups.

3.5.4 Safety assessments

Adverse events were assessed at Day 1, 7 and 14 and recorded in accordance with GCP and local trust policy. Operational definitions are presented in Table 3.5.2.

Operational definitions for (S)AEs

Adverse events were recorded in the CRF. If there was suspicion that the participants' disease has progressed faster due to the administration of an IMP, then it was recorded and reported as an unexpected adverse event. Clinically significant derangements in the results of objective tests (e.g. bloods, spirometry) were recorded as adverse events. Any requirement for a prolonged course of antibiotics

beyond 14 days was recorded as an AE. All adverse events were recorded with clinical symptoms and accompanied with a simple, brief description of the event, including dates as appropriate via the Liverpool Heart and Chest Hospital local AE form. All adverse events were recorded until the day of completion of each trial arm.

Recording and reporting of SAEs AND SUSARs

All SAEs/SUSARs occurring from the time of start of trial treatment until 1-day post cessation of trial treatment were recorded and delivered to the Sponsor within 24 hours or 7 day (of research team becoming aware) for SUSARs and SAEs respectively.

Table 3.5.2: Operational definition for adverse events and adverse reactions in the AZTEC-CF study.

Term	Definition
Adverse Event (AE)	Any untoward medical occurrence in a participant to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product.
Adverse Reaction (AR)	<p>An untoward and unintended response in a participant to an investigational medicinal product which is related to any dose administered to that participant.</p> <p>The phrase "response to an investigational medicinal product" means that a causal relationship between a trial medication and an AE is at least a reasonable possibility, i.e. the relationship cannot be ruled out.</p> <p>All cases judged by either the reporting medically qualified professional or the Sponsor as having a reasonable suspected causal relationship to the trial medication qualify as adverse reactions.</p>
Serious Adverse Event (SAE)	<p>A serious adverse event is any untoward medical occurrence that:</p> <ul style="list-style-type: none"> • results in death • is life-threatening • requires inpatient hospitalisation or prolongation of existing hospitalisation • results in persistent or significant disability/incapacity • consists of a congenital anomaly or birth defect <p>Other 'important medical events' may also be considered serious if they jeopardise the participant or require an intervention to prevent one of the above consequences.</p>
Serious Adverse Reaction (SAR)	An adverse event that is both serious and, in the opinion of the reporting Investigator, believed with reasonable probability to be due to one of the trial treatments, based on the information provided.
Suspected Unexpected Serious Adverse Reaction (SUSAR)	<p>A serious adverse reaction, the nature and severity of which is not consistent with the information about the medicinal product in question set out:</p> <ul style="list-style-type: none"> • in the case of a product with a marketing authorisation, in the summary of product characteristics (SmPC) for that product • in the case of any other investigational medicinal product, in the investigator's brochure (IB) relating to the trial in question

3.6 Outcomes

3.6.1 Primary endpoint

1. Change in absolute % predicted FEV1 from Day 1 to Day 14

3.6.2 Secondary endpoints

1. Days to next protocol-defined pulmonary exacerbation
-Defined as the need for intravenous antibiotics in the presence of 2 or more criteria, see Section 3.4.6.
2. Change in the Cystic Fibrosis Questionnaire-Revised (CFQ-R) Respiratory domain
3. Change in sputum bacterial load from Day 1 to Day 14
4. Change in serum inflammatory markers from Day 1 to Day 14
5. Changes in aztreonam resistance from Day 1 to Day 14
6. Changes in lung microbiota from Day 1 to Day 14

3.7 Protocol amendments

Non-substantial amendment 1

Initially the protocol excluded those people with CF under the age of 18 years. Non-substantial amendment 1 changed the lower age limit from 18 to 16 years old to reflect the fact people with CF transition to the adult clinic at LHCH from the age of 16 years onwards and are able to give fully informed consent. Excluding those 16-18 years of age was therefore deemed inappropriate and the amendment was approved by the HRA on 9th June 2017.

Non substantial amendment 2

The second amendment to the protocol removed a day 28 outcome assessment. Participants enrolled early in the study reported coming back to hospital so soon after a 14-day inpatient stay was unappealing and often not feasible. A number of protocol deviations were logged in response to non-attendance at day 28 visit. Given day 28 spirometry is neither part of routine clinical practice nor a frequently reported as an exacerbation outcome measure, removal of this visit was felt unlikely to impact

the scientific merit, clinical relevance or safety of the trial. The amendment was approved by the HRA on 30th January 2018.

3.8 Statistical considerations

Statistical analyses were conducted in RStudio (v1.0.136, R Studio Inc). After normality testing using the Shapiro-Wilk test, only lung function data was found to be distributed normally. Lung function is therefore presented as mean \pm SD throughout, and all other outcomes are presented as median [IQR]. Between-treatment comparisons e.g. comparison of change in lung function over 14 days between IV+IV and AZLI+IV, were conducted as intention to treat analyses and presented as mean or median difference [95% confidence interval] depending on normality of the outcome variable. Unpaired between-group comparisons were made using t-test or Mann-Whitney U test depending on normality. Paired data were compared using Wilcoxon signed-rank test. Unadjusted *p*-values are presented throughout and a significance level of <0.05 was considered significant.

Chapter 4: Aztreonam Lysine for the Treatment of Exacerbations of CF: An Open-Label Pilot Randomised Crossover Study (AZTEC-CF)

4.1 Introduction

The AZTEC-CF protocol is described in detail in Chapter 3. The Health Research Authority approved AZTEC-CF in December 2016 and the study opened for recruitment in January 2017. Three participants were withdrawn from the study prior to randomisation, see Figure 4.2.1. One participant withdrew consent, one participant no longer met the inclusion/exclusion criteria as they were commenced on chronic AZLI therapy prior to randomisation and one participant failed to attend for AZLI test dose and was therefore withdrawn from the study by the PI. A timeline of exacerbations and designated treatments is set out in Figure 4.2.2.

4.2 Results

4.2.1 Study completion

All 16 randomised participants successfully completed the first study arm. Two participants withdrew prior to their second exacerbation and a further two did not exacerbate for a second time before study censorship in February 2019. Thus, 28 of a possible 32 exacerbations (87.5%) were completed and included for analysis. Of these, 24 exacerbations (12 pairs from participants who completed both treatment arms) were available for all statistical calculations.

4.2.2 IV+IV arm antibiotics

During the IV+IV arm, participants received intravenous colistimethate 2 Megaunits three times a day (TDS) along with another IV antibiotic selected on clinical grounds by the admitting team, see Table 4.2.1.

Table 4.2.1: Second intravenous anti-pseudomonal agent used in the IV+IV arm.

Antibiotic	Dose	Used, <i>n</i> (%)
Meropenem	1g TDS	9 (56)
Ceftazidime	3g TDS	6 (38)
Piperacillin/Tazobactam	4.5g TDS	1 (6)

TDS= Thrice daily dosing

4.2.3 Study participants

Baseline demographics and clinical characteristics are presented in Table 4.2.2. Briefly, the median [IQR] age was 29.5 years [24.5 to 32.5], mean \pm SD FEV1 was 52.4 % \pm 14.7, median BMI was 22.1 kg/m² [19.2 to 23.6] and participants had received a median 26 days [13.8 to 45.8] of intravenous antibiotics in the preceding 12 months. No participants had ever received CFTR modulators. 10/16 (62.5%) were infected with the transmissible Liverpool Epidemic Strain of *P. aeruginosa*, 4/16 (25.0%) were co-infected with *S. aureus* and 3/16 (18.8%) co-infected with *Aspergillus* spp. Despite males and females being distributed evenly within the LHCH clinics from which participants were recruited, only one female study participant was recruited and consented meaning the study cohort was predominantly male. At enrolment, 15/16 (93.7%) participants were receiving an inhaled anti-pseudomonal antibiotic regimen: 10/16 (62.5%) continuous inhaled colistimethate preparations, 4/16 (25.0%) monthly alternating inhaled colistimethate and tobramycin preparations, and 1/16 (6.3%) alternate month tobramycin monotherapy alone. Chronic oral macrolide therapy was prescribed in 16/16 (100.0%). To allow comparison of clinical parameters between exacerbations, participant level data from day 1 of each exacerbation is presented in Table 4.2.3. Overall, there were no meaningful differences in median day 1 parameters across exacerbations and on an individual level there was evidence that inflammatory markers were similar across exacerbations given 75% of those with raised CRP at exacerbation 1, also had a raised CRP at exacerbation 2. Similarly, 87.5% of those with normal CRP at exacerbation 1 also had normal CRP at exacerbation 2.

Figure 4.2.1: CONSORT Diagram for AZTEC-CF Study

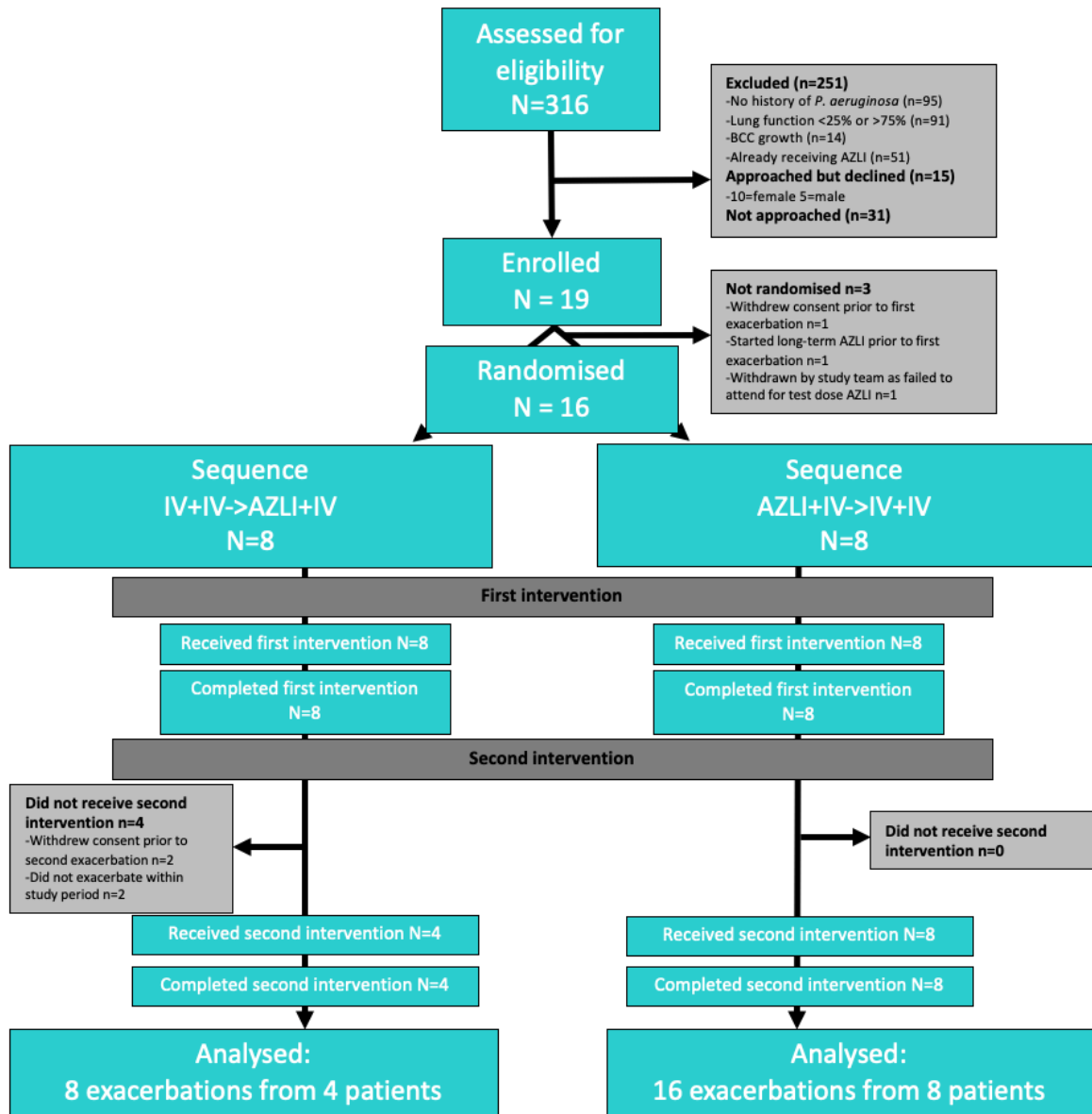


Figure 4.2.2: Timeline of exacerbation events for each study participant

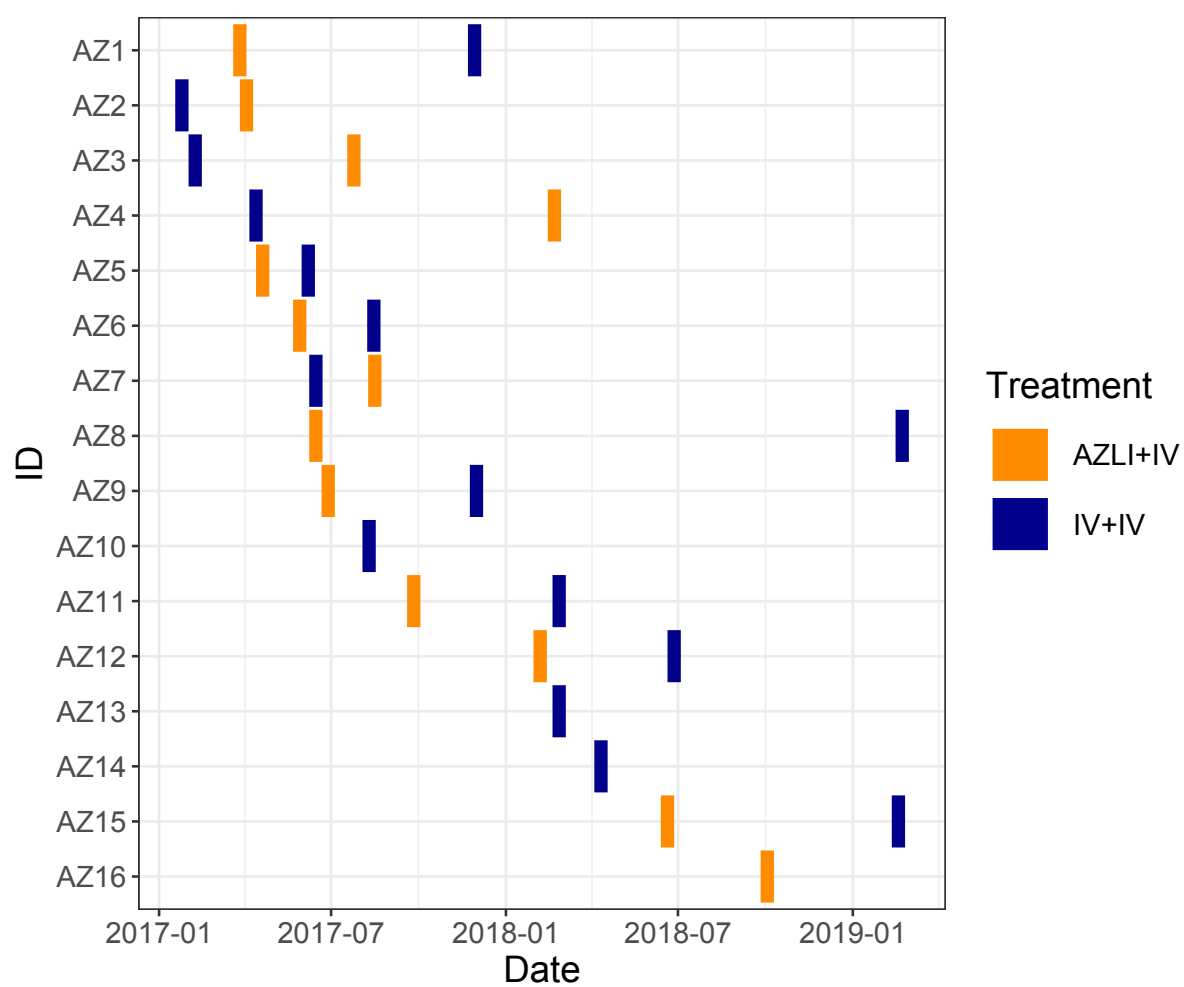


Table 4.2.2: Clinical characteristics of participants in the AZTEC-CF study, with comparison by allocated sequence.

Data presented as n (%), median [IQR] or mean \pm SD.

	AZLI+IV/IV+IV	IV+IV/AZLI+IV	All
Demographics			
Age	28.9 [25.4, 30.3]	29.7 [22.4, 33.1]	29.5 [24.5, 32.5]
Sex, <i>male</i>	7 (87.5)	8 (100)	15 (93.8)
Phe508del homozygous	8 (100)	3 (7.5)	11 (68.8)
Phe508del heterozygous	0 (0.0)	5 (62.5)	5 (31.2)
FEV1, % <i>predicted</i>	47.9 \pm 14.4	56.9 \pm 14.5	52.4 \pm 14.7
BMI, <i>kg/m</i> ²	22.6 [21.2, 25.0]	20.0 [19.1, 22.3]	22.1 [19.2, 23.6]
Annualised IV days	26.0 [20.0, 35.3]	20.50 [6.8, 45.8]	26.0 [13.8, 45.8]
Pancreatic insufficiency	6 (75.0)	8 (100.0)	14 (87.5)
GORD	5 (62.5)	6 (75.0)	11 (68.8)
CFRD	6 (75.0)	5 (62.5)	11 (68.8)
Microbiology			
<i>P. aeruginosa</i>	8 (100.0)	8 (100.0)	16 (100.0)
Liverpool Epidemic Strain	5 (62.5)	5 (62.5)	10 (62.5)
<i>S. aureus</i>	4 (50.0)	0 (0.0)	4 (25.0)
NTM			
<i>M. avium</i> complex	0 (0.0)	1 (12.5)	1 (6.2)
<i>M. abscessus</i> complex	1 (12.5)	2 (25.0)	3 (18.8)
<i>Aspergillus</i> spp.	2 (25.0)	1 (12.5)	3 (18.8)
Long-term oral medication			
Macrolide	8 (100.0)	7 (87.5)	15 (93.8)
Proton pump inhibitor	8 (100.0)	4 (57.1)	12 (80.0)
Ursodeoxycholic acid	6 (75.0)	4 (50.0)	10 (62.5)
Dornase Alfa	6 (75.0)	6 (75.0)	12 (75.0)
Nebulised therapies			
Colistimethate	6 (75.0)	4 (50.0)	10 (62.5)
Tobramycin	1 (12.5)	0 (0.0)	1 (12.5)
Both	0 (0.0)	4 (50.0)	4 (25.0)

Abbreviations: FEV1=Forced expiratory volume in 1 second; BMI=Body Mass Index;

GORD=Gastro-oesophageal reflux disease; CFRD=Cystic fibrosis related diabetes;

NTM=Non-tuberculous mycobacteria

Table 4.2.3: Participant level data for Day 1 clinical variables across exacerbation 1 and 2.

Study ID	Lung function: Absolute Drop in % predicted FEV1 from best in previous 6-months		Day 1 C-reactive protein mg/L		Day 1 White Cell Count $10^9/ml$		Day 1 CFQ-R Respiratory Domain	
	Exac. 1	Exac. 2	Exac. 1	Exac. 2	Exac. 1	Exac. 2	Exac. 1	Exac. 2
1	-19	-28	39	47	12.9	17.8	38.89	44.44
2	-20	-16	9	4	11.6	12.6	33.33	44.44
3	-21	-30	4	6	7.4	13.9	55.56	55.56
4	-20	-17	4	5	4.5	4.9	61.11	55.56
5	-13	-13	21	69	12.6	15.3	11.11	44.44
6	-13	-22	26	68	10.8	10.6	27.78	22.22
7	-46	-41	4	4	5.1	13.3	50.00	61.11
8	-5	-4	4	7	12	11.3	61.11	44.44
9	-8	-17	4	4	13.1	7.4	27.78	38.89
10	-6		4		5.9		66.67	
11	-13	-9	40	7	22.3	13.9	72.22	83.33
12	-11	-5	8	25	11.9	14.8	55.56	22.22
13	-3		95		31.0		33.33	
14	-6		34		14.4		22.22	
15	-11	8	9	4	11.5	8.3	61.11	66.67
16	-11		4		7.6		27.78	
Median	-13	-16.5	9	6.5	11.9	12.95	50.00	44.44

4.3.1 Adjunct therapies

Other routine CF treatments that might influence clinical response during an exacerbation (adjunct therapies) are presented in Table 4.3.1 & Table 4.3.2. Comparison of these adjunct therapies confirmed no intrinsic differences between treatment arms, see Table 4.3.3.

4.3.2 No evidence of carry-over effect between exacerbations

A “carry-over” effect can bias estimation of treatment effects in cross-over studies. To assess for any such effect, the average sum of day 14 improvements were compared between participants randomised to AZLI+IV followed by IV+IV (Group AB) and those randomised to IV+IV followed by AZLI+IV (Group BA) as previously described. [288] The null hypothesis tested here is that carry-over effects are equal between groups and results are presented in Table 4.3.4. No significant differences were observed between allocation groups for any of the outcomes of interest, meaning no evidence of carry-over effect acting as a source of bias was found.

Table 4.3.1: Adjunct therapies - AZLI+IV Arm

Abbreviation: HTS=Hypertonic saline; SCTI=Subcutaneous terbutaline infusion

ID	Allocation	Treatment Arm A	Treatment Arm B		Treatment adjunct										
					HTS	Dornase	Prednisolone	Dose (mg)	Theophylline	SCTI	Macrolide				
1	AB	AZLI+Colistimethate	Ceftazidime	Colistimethate	Y	Y		Y	15		N	Y		Y	
2	BA	AZLI+Colistimethate	Meropenem	Colistimethate	N	Y			Y	30		N	Y		Y
3	BA	AZLI+Colistimethate	Tazocin	Colistimethate	Y	N			Y	30		N	N		Y
4	BA	AZLI+Colistimethate	Meropenem	Colistimethate	Y	Y			Y	30		N	Y		Y
5	AB	AZLI+Colistimethate	Meropenem	Colistimethate	Y	Y			Y	20		N	Y		Y
6	AB	AZLI+Colistimethate	Meropenem	Colistimethate	Y	Y			Y	30		N	N		Y
7	BA	AZLI+Colistimethate	Meropenem	Colistimethate	Y	Y			Y	30		Y	Y		Y
8	AB	AZLI+Colistimethate	Meropenem	Colistimethate	N	Y			Y	30		N	Y		Y
9	AB	AZLI+Colistimethate	Meropenem	Colistimethate	Y	Y			Y	30		N	Y		Y
10	BA	AZLI+Colistimethate	Meropenem	Colistimethate	Subject did not exacerbate second time within study period										
11	AB	AZLI+Colistimethate	Ceftazidime	Colistimethate	N	Y			Y	30		N	Y		Y
12	AB	AZLI+Colistimethate	Ceftazidime	Colistimethate	Y	Y			Y	30		Y	Y		Y
13	BA	AZLI+Colistimethate	Ceftazidime	Colistimethate	Subject did not exacerbate second time within study period										
14	BA	AZLI+Colistimethate	Meropenem	Colistimethate	Subject withdrew										
15	AB	AZLI+Colistimethate	Ceftazidime	Colistimethate	N	Y			N	0		N	Y		Y
16	BA	AZLI+Colistimethate	Ceftazidime	Colistimethate	Subject withdrew										

Table 4.3.2: Adjunct therapies - IV+IV Arm

Abbreviation: HTS=Hypertonic saline; SCTI=Subcutaneous terbutaline infusion

ID	Treatment		Treatment adjunct						
			HTS	Dornase	Prednisolone	Dose (mg)	Theophylline	SCTI	Macrolide
1	Ceftazidime	Colistimethate	Y	Y	Y	30	N	Y	Y
2	Meropenem	Colistimethate	N	Y	Y	30	N	Y	Y
3	Tazocin	Colistimethate	N	N	Y	30	N	N	Y
4	Meropenem	Colistimethate	Y	Y	Y	30	N	Y	Y
5	Meropenem	Colistimethate	Y	Y	Y	30	N	Y	Y
6	Meropenem	Colistimethate	Y	Y	Y	30	N	N	Y
7	Meropenem	Colistimethate	Y	Y	Y	30	Y	Y	Y
8	Meropenem	Colistimethate	Y	Y	Y	30	N	Y	Y
9	Meropenem	Colistimethate	Y	Y	Y	30	N	Y	Y
10	Meropenem	Colistimethate	Y	Y	Y	30	N	Y	Y
11	Ceftazidime	Colistimethate	N	Y	Y	30	N	Y	Y
12	Ceftazidime	Colistimethate	N	Y	Y	30	Y	N	Y
13	Ceftazidime	Colistimethate	Y	Y	Y	30	Y	Y	Y
14	Meropenem	Colistimethate	Y	Y	Y	30	N	Y	Y
15	Ceftazidime	Colistimethate	N	Y	Y	30	N	Y	Y
16	Ceftazidime	Colistimethate	Y	Y	N	0	N	N	Y

Table 4.3.3 Comparison of acute adjunct and chronic therapies prescribed during each exacerbation.

Data are presented as n (%) or median [IQR]

	AZLI+IV	IV+IV
n	12	16
Acute adjuncts		
Nebulised bronchodilators	12 (100%)	16 (100%)
Oral corticosteroid	11 (91.7)	15 (93.8)
Dose, <i>mg</i>	30.0 [27.5, 30.0]	30.0 [30.0, 30.0]
Aminophylline infusion	2 (16.7)	3 (18.8)
Subcutaneous terbutaline infusion	10 (83.3)	12 (75.0)
Chronic therapies		
Azithromycin	12 (100.0)	16 (100.0)
Hypertonic saline	8 (66.7)	11 (68.8)
Dornase alfa	11 (91.7)	15 (93.8)
Initiated during admission	0 (0.0)	2 (12.5)

Table 4.3.4 Mean differences between sums of Day 14 measurements between groups AB (AZLI+IV followed by IV+IV) and BA (IV+IV followed by AZLI+IV)
Difference and 95% confidence intervals are presented.

Day 14 outcome	Differences	95% CI	<i>p</i> -value
Systemic inflammatory markers			
White blood cell count	2.45	-12.6 to 17.5	0.68
Neutrophils	2	-12.7 to 16.7	0.74
Platelets	20	-147 to 187	0.78
C-Reactive Protein	5.375	-1.58 to 12.33	0.11
Change in White blood cell count	0.85	-10.4 to 12.1	0.86
Change in neutrophils	0.91	-6.54 to 8.36	0.78
Change in platelets	-21.85	-125.6 to 81.9	0.63
Change in C-reactive protein	20.75	-26.3 to 67.8	0.32
Lung function			
FEV1 (% predicted)	-11.5	-46.8 to 23.8	0.46
Change in FEV1 (% predicted)	-10.875	-28.6 to 6.85	0.18
Change in Fev1 (litres)	-0.501	-1.237 to 0.235	0.14
Quality of life			
CFQ-R Respiratory Domain	0.7	-52.5 to 53.9	0.98
Change in CFQ-R Respiratory Domain	-6.95	-45.8 to 31.9	0.69

4.3.4 AZLI+IV and IV+IV are both associated with improved lung function over 14 days

On day 1, lung function was similar between AZLI+IV (mean \pm SD % predicted FEV1 50.4% \pm 12.5) and IV+IV (53.1% \pm 17.4), mean [95% CI] difference between groups -2.6 [-15.0 to 9.0], $p=0.46$. Equally, on day 1, decline in % predicted FEV1 from previous 6-month best was similar between groups (-18.4% for AZLI+IV and -14.4% for IV+IV, mean [95% CI] difference 4.0 [-5.2 to 13.2], $p=0.37$). After 7 days treatment with AZLI+IV, lung function had significantly increased (mean [95% CI] absolute improvement in % predicted FEV1: +9.6% [3.2 to 16.2], $p=0.007$). After 14 days of treatment, lung function had improved +13.5% [6.5 to 20.5], $p=0.001$, see Figure 4.3.1A. In the IV+IV group, significant improvements from baseline were also observed such that by day 7, FEV1 had improved +7.9% [1.6 to 14.3], $p=0.02$ and by day 14 mean improvement was +8.3% [3.2 to 13.4], $p=0.003$, see Figure 4.3.1B.

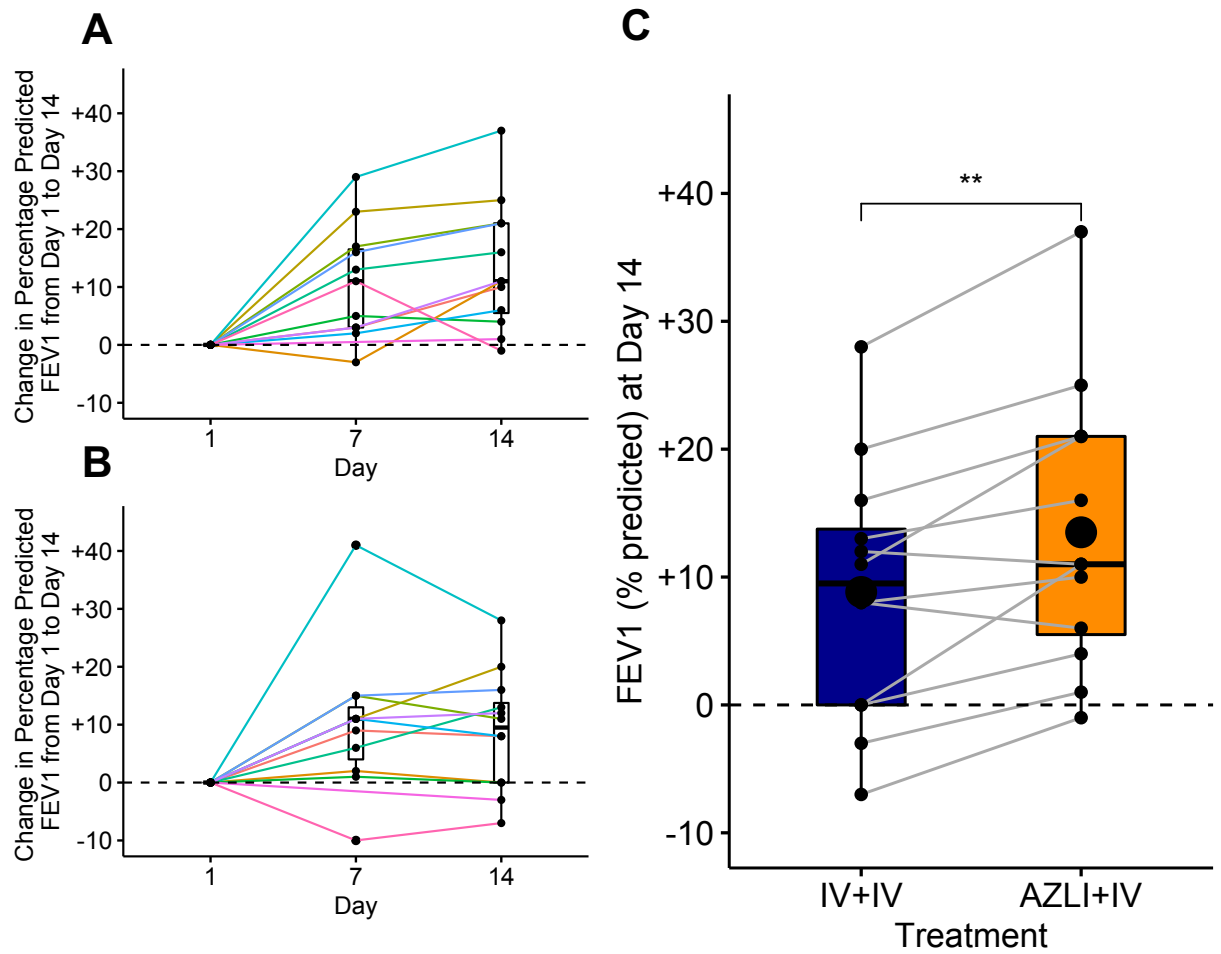
4.3.5 Primary outcome:

4.3.5.1 AZLI+IV is associated with greater lung function improvement than IV+IV at day 14

Participants whom completed both treatment arms were next included in a paired analysis for the primary outcome. After 14 days of treatment, AZLI+IV was associated with significantly greater lung function improvement from baseline than IV+IV (+13.5% \pm 11.0 vs. +8.8% \pm 10.1, mean [95% CI] paired treatment difference +4.6% [2.1 to 7.2], $p=0.002$), see Figure 4.3.1.

Figure 4.3.1: Absolute change in % predicted FEV1 for AZLI+IV (A) and IV+IV (B) and paired between-treatment comparison at day 14 (C).

Data are presented as box and whisker plots with lines linking individual study participants' outcomes. **= $p < 0.01$

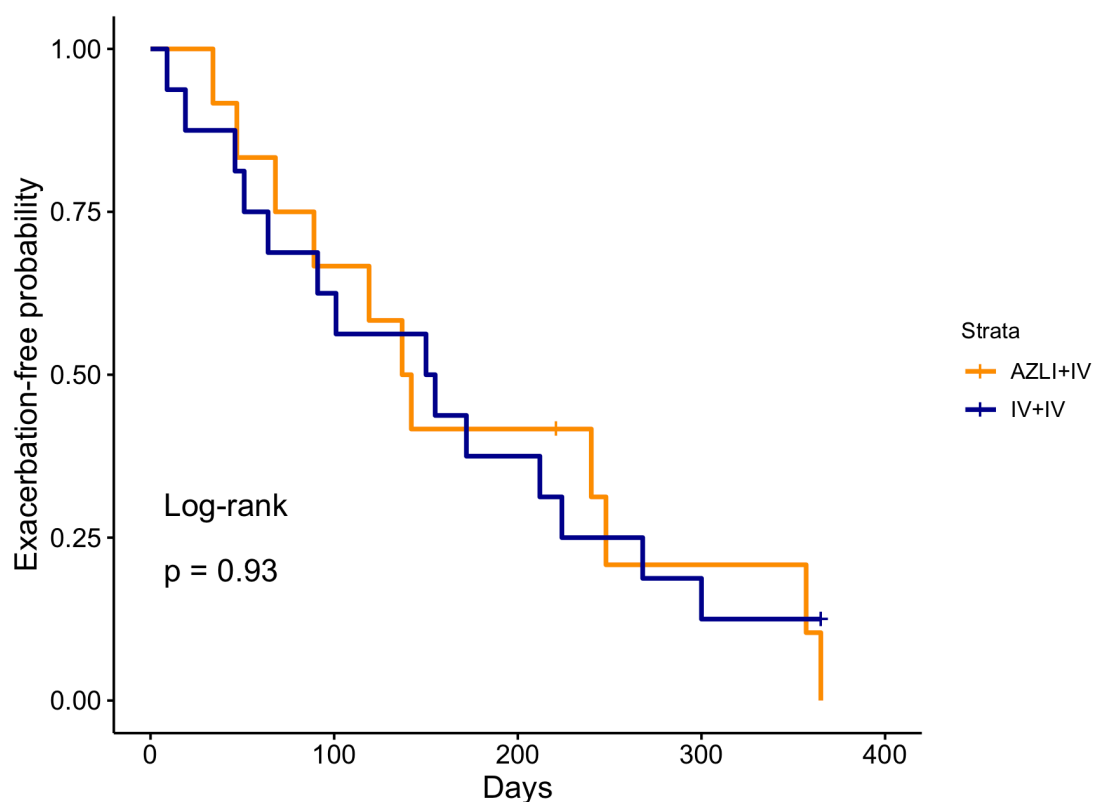


4.3.6 Secondary outcomes

4.3.6.1 Similar time to next exacerbation for each treatment

Time to next exacerbation was similar between treatments. For the IV+IV treatment median (95% confidence interval) time to next exacerbation was 152 days [64-300] and for the AZLI+IV treatment 140 days [89-318], log-rank $p = 0.93$, see Figure 4.3.2

Figure 4.3.2: Kaplan-Meier plot for time to next exacerbation after IV+IV (Blue) and AZLI+IV (Orange).



4.3.6.2 No difference between treatments for changes in systemic inflammatory markers

White blood cells

Median [IQR] serum white blood cell counts were similar between groups at treatment initiation (AZLI+IV arm: 12.3 [11.3, 13.0] $\times 10^9/\text{ml}$, IV+IV arm: 11.8 [7.6, 14.5] $\times 10^9/\text{ml}$, $p=0.98$) and leucocytosis was present in 9/16 (56.3%) and 9/12 (75.0%) participants receiving IV+IV and IV+AZLI respectively ($p=0.34$). Neither treatment was associated with significant differences in white blood cell count between day 1 and day 14 and there was no significant difference between treatments (median [95% CI] paired difference at day 14: 0.4 [-3.1, 4.5] $\times 10^9/\text{ml}$, $p=0.73$), see Figure 4.3.3. Of those participants with leucocytosis on day 1, two participants in each arm had a normal white blood cell count at day 14, ($p>0.99$)

C-reactive protein

On day 1, median [IQR] CRP was equal between groups (AZLI+IV: 7.0 mg/L [<5.0 , 22.3] vs. IV+IV: 7.0 mg/L [<5.0 , 40.5], $p=0.6$) and an elevated CRP was present in 7/12 (58.3%) and 9/16 (50.0%) participants in the AZLI+IV and IV+IV groups respectively. Paired samples were available for 10/12 participants completing both treatments. Of those with an elevated CRP on Day 1, 4/7 (57.1%) in the AZLI+IV group had normal CRP at Day 14 compared to 7/9 (77.8%) in the IV+IV group, $p=0.44$. There were no significant differences in changes in CRP at day 7 or day 14 overall (median [95% CI] difference 4.0 [-9 to 26], $p=0.40$), see Figure 4.3.4.

When only those with a raised CRP on day 1 were considered, median [IQR] day 1 CRP was 34 mg/L [9 to 68] for the IV+IV group and 21 mg/L [8 to 39] for the AZLI+IV group. After 7 days of treatment, CRP had fallen to 15 mg/L and 10 mg/L respectively, median [95% CI] differences between groups 17 mg/L [-6 to 45], $p=0.15$. After 14 days of treatment, median CRP in both groups was <5 mg/L and although reductions in CRP at day 14 were numerically greater in the IV+IV, there were no statistically significant differences in either change in CRP or rates of normalisation of CRP at day 14.

Figure 4.3.3: Paired changes in white blood cell count ($10^9/\text{ml}$) after 14 days treatment with IV+IV (blue) and AZLI+IV (orange).

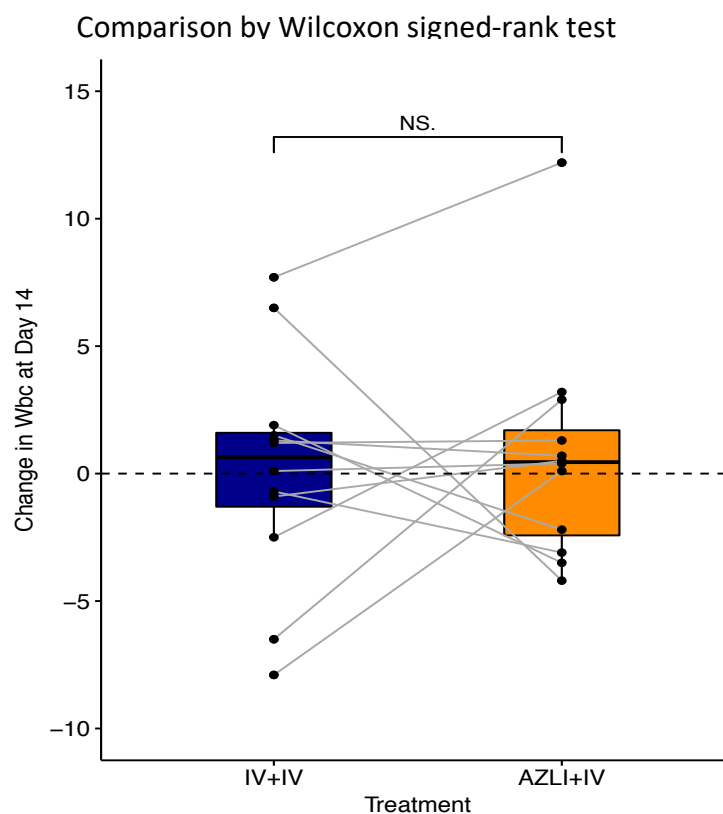
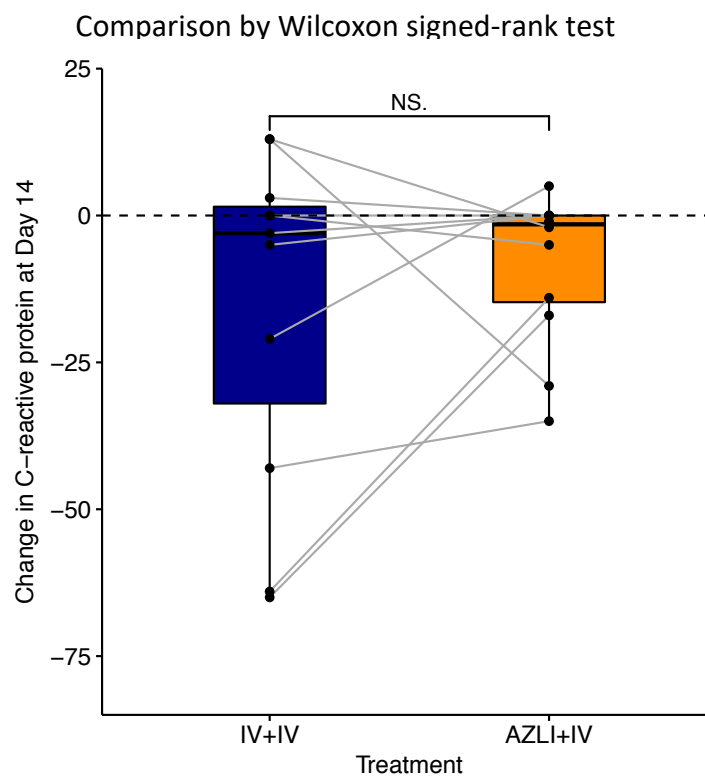


Figure 4.3.4: Paired changes in CRP (mg/L) after 14 days treatment with IV+IV (blue) and AZLI+IV (orange).



4.3.6.3 Relevant improvements in quality of life achieved more often with AZLI+IV

The Cystic Fibrosis Questionnaire-Revised (CFQ-R) score is a health-related quality of life tool validated in the CF setting. During pulmonary exacerbations, a minimum clinically important difference (MCID) has been validated as 8.5 points for the CFQ-R Respiratory domain. [291] AZLI+IV was associated with median [IQR] improvement in CFQ-R Respiratory domain scores of +11.1 [11.1, 18.1]. This change was not significantly different from that seen in the IV+IV arm where a median improvement of +8.3 [-1.4, 18.1] was observed, median paired differences 8.3 [-22.2 to 33.3], $p=0.73$ see Figure 4.3.5. However, the validated MCID was achieved more frequently with AZLI+IV where 10/12 (83.3%) participants achieved an improvement in CFQ-R Respiratory Domain of ≥ 8.5 points, compared with 7/16 (43.8%) in the IV+IV arm, Fisher's exact test $p=0.04$.

4.3.6.4 Similar safety profiles between treatments

Adverse events were reported during the study in 10/16 (62.5%) participants. The proportion of participants experiencing an adverse event was identical between AZLI+IV and IV+IV treatment periods respectively: 6/12 (50%) vs. 8/16 (50%), $p>0.99$. All adverse events are presented in Table 4.3.5. The most common adverse event was the need for prolonged antibiotics beyond 14 days of trial treatment which occurred in 7/28 (25%) admissions and was seen similarly in the AZLI+IV and the IV+IV treatment periods: 3/12 (25.0%) and 4/16 (25.0%) respectively. Other treatment-emergent adverse events seen similarly across both treatment groups included rise in serum C-reactive protein (AZLI+IV: 1/12 (8.3%) vs. IV+IV: 2/16 (12.5%), $p=0.39$) and pyrexia (AZLI+IV: 2/12 (16.7%) vs. IV+IV: 1/16 (6.3%), $p=0.22$). Aggravated cough was reported by 2/12 (16.7%) participants during the AZLI+IV treatment but by 0/16 (0%) during the IV+IV arm ($p=0.16$), whereas headache was reported in 2/16 (12.5%) participants during the IV+IV arm and 0/16 (0%) during the AZLI+IV arm ($p=0.17$).

**Figure 4.3.5: Paired changes in CFQ-R Respiratory Domain for IV+IV (blue) and
Comparison by Wilcoxon signed-rank test**

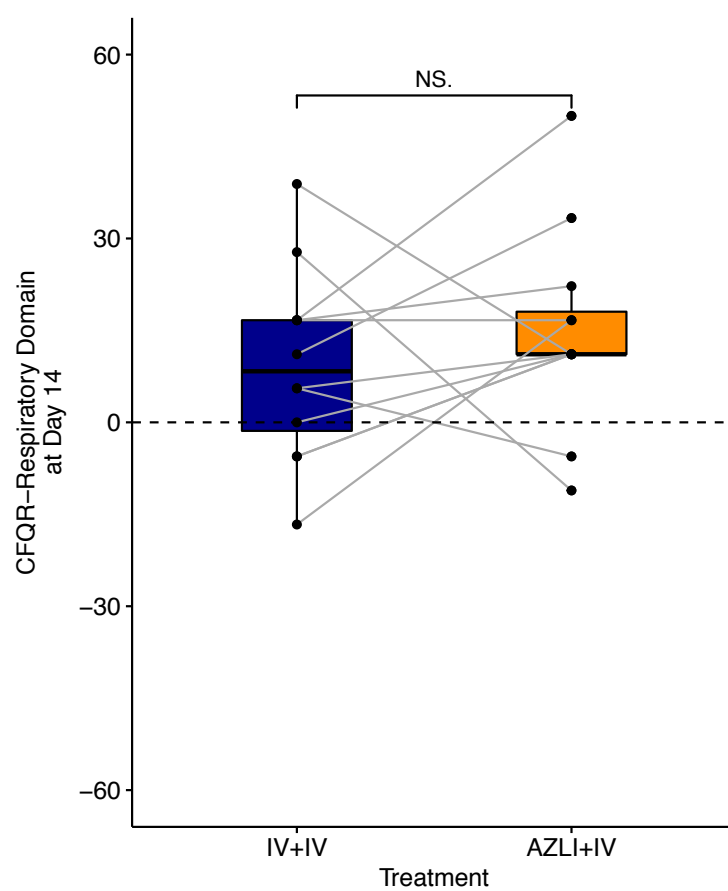


Table 4.3.5: Treatment emergent adverse events in AZTEC-CF studyComparison by Fisher's exact test. Not significant (NS)= $p > 0.05$

	AZLI+IV (n=12) %		IV+IV (n=16) %		P
Total patients reporting an adverse event	6	50.0%	8	50.0%	0.70
Adverse event					
>14 days antibiotic therapy required	3	25.0%	4	25.0%	>0.99
Drop in lung function	1	8.3%	0	0.0%	0.23
Pyrexia	2	16.7%	1	6.3%	0.38
Raised inflammatory markers	1	8.3%	2	12.5%	0.72
Headache	0	0.0%	2	12.5%	0.20
Cough	2	16.7%	0	0.0%	0.10
Nausea	0	0.0%	1	6.3%	0.38
Bloating	1	8.3%	0	0.0%	0.23
Musculoskeletal pain	0	0.0%	1	6.3%	0.38

4.3.7 Exploratory baseline characteristic subgroup analysis

A number of exploratory subgroup analyses were undertaken in order to assess whether any baseline characteristics were associated with different treatment outcomes. Caution must be taken when interpreting these findings due to low numbers included in these analyses.

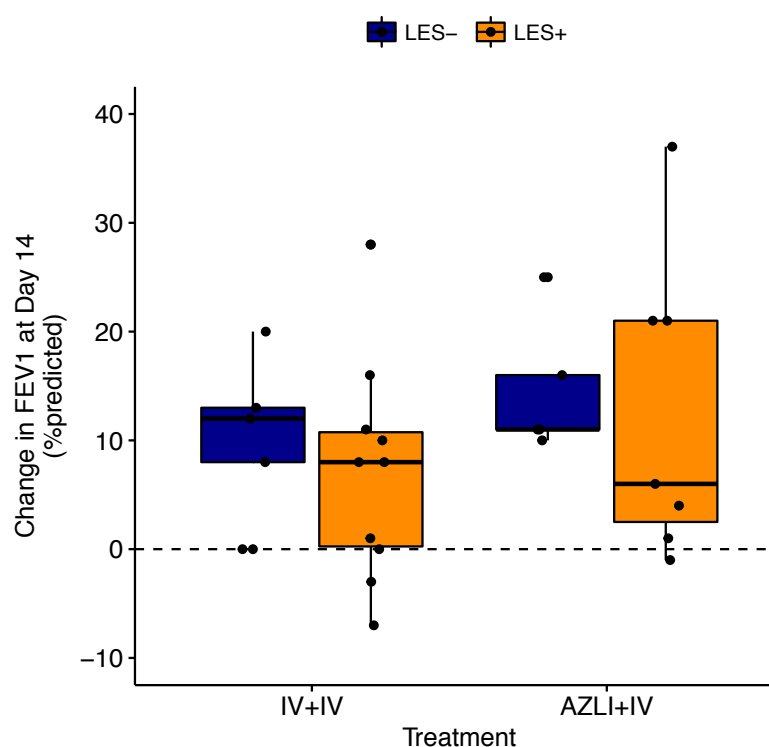
A two-way analysis of variance tested the interaction between LES status and change in lung function for each treatment. No significant interaction for LES on treatment responses observed, see Figure 4.3.6 and Table 4.3.6. The influence of co-colonising species on treatment outcomes was examined by testing the effect of recognised CF pathogens *S. aureus*, *Aspergillus spp.* and NTM on changes in lung function at day 14. For the purposes of this analysis co-colonisation was considered as at least one growth of that organism within the previous 12 months. Additionally, further analysis including the presence of ABPA was also undertaken. No effects on treatment outcomes were observed for either the co-colonising pathogens or a diagnosis of ABPA, see Figure 4.3.7 and Table 4.3.7.

Table 4.3.6 Two-way analysis of variance between lung function changes at day 14, treatment arm and Liverpool epidemic strain

	Df	Sum Squares	Mean Squares	F	p
Treatment	1	178	178	1.7	0.21
LES	1	45	45	0.4	0.52
Interaction	1	4	4	0.03	0.86

Figure 4.3.6: Comparison of changes in lung function between treatment groups based on Liverpool Epidemic Strain status.

Comparison by Wilcoxon signed-rank test



4.3.7.1 Elevated systemic inflammatory markers at admission

This subgroup analysis focussed on whether different treatment efficacy was seen between those participants with and without raised inflammatory markers at admission. Overall, an elevated C-reactive protein at admission was associated with poorer improvement in lung function at day 14 (mean difference 9.0% predicted FEV1 [0.0 to 17.0], $p=0.05$). However this pattern was seen in both treatment groups, see Figure 4.3.8, and two-way analysis of variance showed no evidence of an interaction between C-reactive protein and Treatment group, see Table 4.3.8. Similarly, an elevated white blood cell count at admission was associated with poorer lung function improvements at day 14 (mean difference 9.9% [0.1 to 17.1], $p=0.05$) and this effect was similar across groups, Figure 4.3.8 and Table 4.3.9. Taken together these results suggest participants with raised systemic inflammatory markers on Day 1 saw less lung function improvement after fourteen days regardless of which treatment they received.

Table 4.3.7: Two-way analysis of variance between lung function changes at day 14, treatment arm and sputum microbiology

	Df	Sum Squares	Mean Squares	F	p
Treatment	1	178	178	1.8	0.19
<i>S. aureus</i>	1	262	262	2.7	0.12
Interaction	1	16	16	0.2	0.69

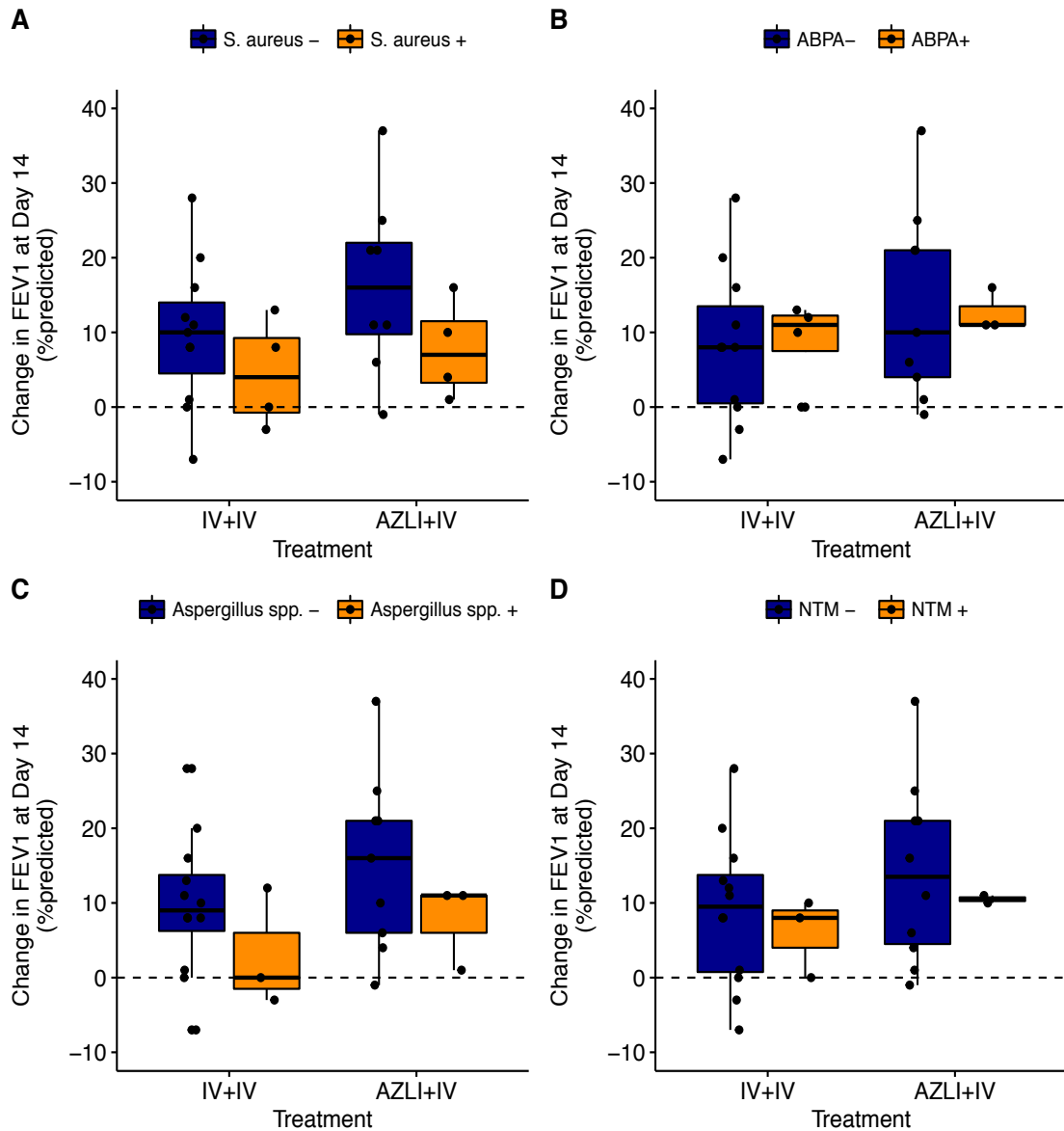
	Df	Sum Squares	Mean Squares	F	P
Treatment	1	178	178	1.6	0.22
ABPA	1	0.1	0.1	0.1	0.97
Interaction	1	3.6	3.6	0.03	0.86

	Df	Sum Squares	Mean Squares	F	p
Treatment	1	178	178	1.8	0.20
<i>Aspergillus</i> spp.	1	241.3	241.3	2.4	0.13
Interaction	1	1.4	1.4	0.01	0.91

	Df	Sum Squares	Mean Squares	F	P
Treatment	1	178	178	1.6	0.21
NTM	1	41.6	41.6	0.4	0.54
Interaction	1	0.5	0.5	0.01	0.95

Abbreviations: ABPA=Allergic bronchopulmonary aspergillosis. NTM=Non-tuberculous mycobacteria

Figure 4.3.7: Comparison of change in lung function based on history of positive sputum culture for *S. aureus* (A), *Aspergillus* spp. (C) and NTM (D), or history of ABPA (B).



Abbreviations: ABPA=Allergic bronchopulmonary aspergillosis. NTM=Non-tuberculous mycobacteria

Table 4.3.8: Two-way analysis of variance between lung function changes at day 14, treatment arm and elevated C-reactive protein (CRP)

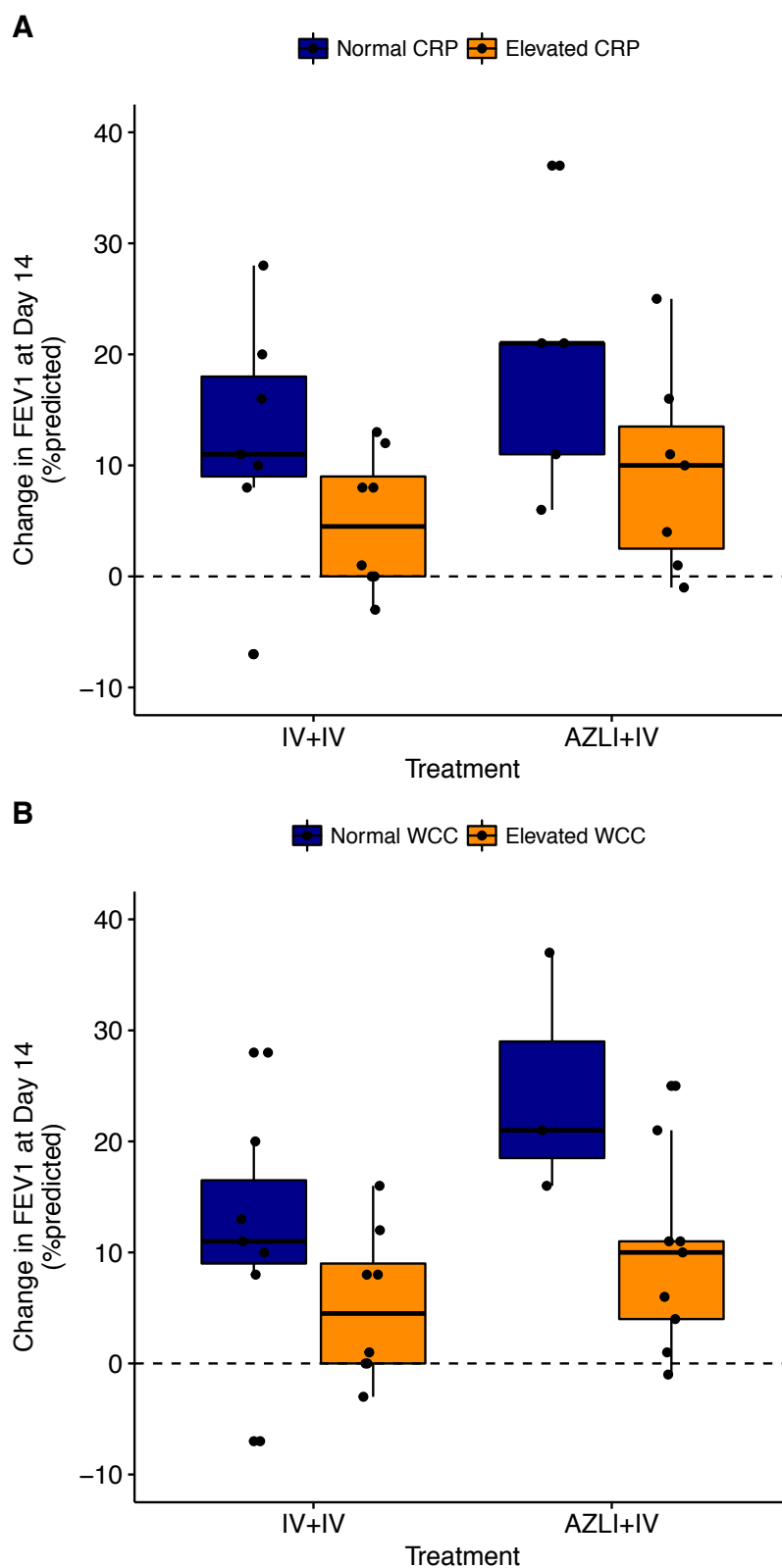
	Df	Sum Squares	Mean Squares	F	p
Treatment	1	178	178	2.0	0.17
Elevated CRP	1	474	474	5.3	0.03
Interaction	1	9	9	0.1	0.75

Table 4.3.9: Two-way analysis of variance between lung function changes at day 14, treatment arm and elevated White blood cell count (WCC)

	Df	Sum Squares	Mean Squares	F	p
Treatment	1	178	178	2.2	0.15
Elevated WCC	1	566	566	7.0	0.01
Interaction	1	96.3	96.3	1.2	0.29

Figure 4.3.8: Comparison of changes in lung function between treatment groups based on C-reactive protein (A) and white blood cell count (B) at treatment initiation on Day 1.

CRP=C-reactive protein; WCC=White blood cell count



4.3.8 Primary outcome sensitivity analyses

4.3.8.1 Primary outcome is robust regardless of FEV1 reporting method

Potential bias exists in the reporting of lung function improvements in terms of absolute changes of % predicted FEV1. Whilst reporting in this manner is common, and easily interpretable for clinicians and people with CF alike, a 10% absolute improvement in FEV1 is less clinically significant for those with a baseline FEV1 of 80% predicted than those with a baseline FEV1 of 40% predicted, where it would represent a proportional increase of 12.5% and 25% respectively. In order to establish whether differences in day 1 FEV1 measurements could have biased results, three further analyses were undertaken. [292,293]

Firstly, a day 1, between-treatment comparison was undertaken for FEV1 expressed in terms of volume and also % of predicted. No significant differences were observed at baseline between AZLI+IV and IV+IV for FEV1 expressed in litres (2.6 ± 0.7 vs. 2.6 ± 0.91 , $p=0.69$) or as percentage of predicted FEV1 ($63.9\% \pm 16.0$ vs. $63.3\% \pm 21.7$, $p=0.82$), see Figure 4.3.9A and Figure 4.3.9B. Secondly, a two-way analysis of covariance (ANCOVA) was performed to assess for an interaction between absolute changes in % predicted FEV1 at day 14 and day 1 FEV1. No significant effect for baseline FEV1 on change in FEV1 at day 14 was seen once controlled for treatment arm (F-statistic 0.63 on 3 and 23 degrees of freedom, $p=0.60$).

Finally, changes in FEV1 at day 14 were expressed as a proportion of FEV1 on day 1, such that a comparison of relative, rather than absolute improvements in lung function could be performed. At day 14, IV+IV was associated with a relative $19.8\% \pm 22.9$ improvement in FEV1 from day 1, compared to $29.3\% \pm 25.1$ improvement for AZLI+IV (mean differences 9.5% [1.3 to 17.7], $p=0.03$), see Figure 4.3.9C. Taken together, the results of these analyses confirm the superior absolute FEV1 (% predicted) improvements associated with AZLI+IV in the primary analyses are not as a result of differing baseline lung function and are robust when considered as both absolute and relative differences.

4.3.8.2 Primary outcome analysis is robust in a per-protocol analysis

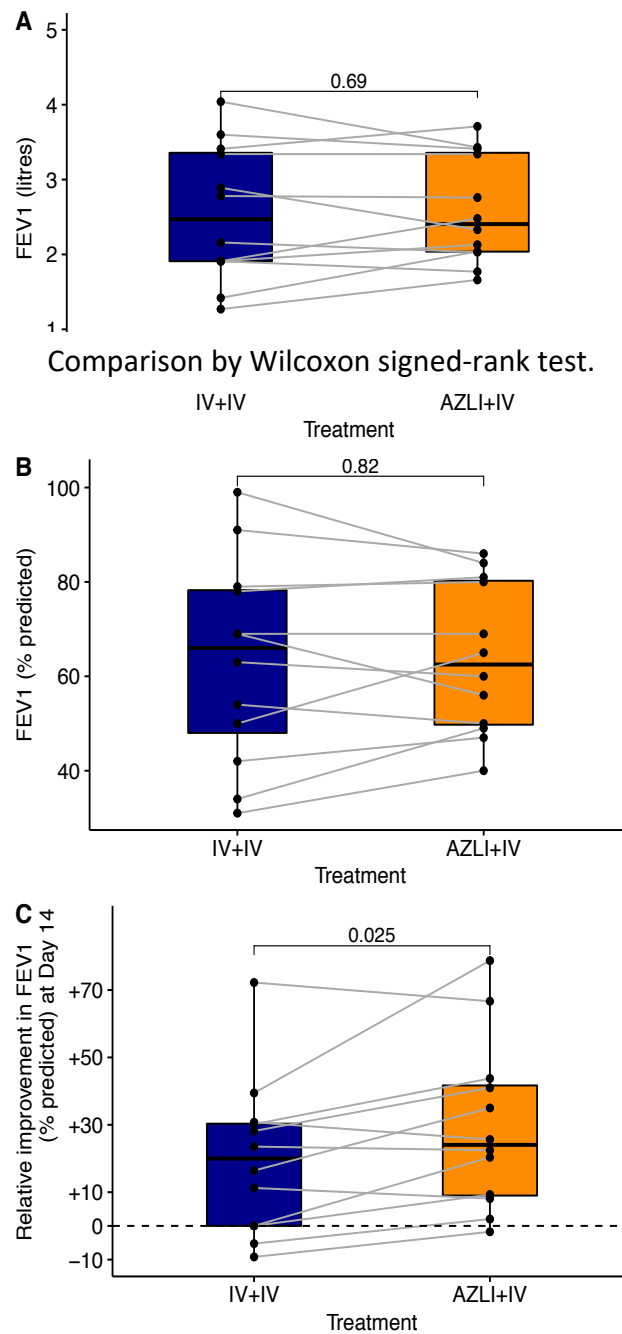
There was a need for further antibiotic treatment beyond 14 days for 3 participants after the AZLI+IV treatment and 4 participants after the IV+IV treatment. To test whether the primary analysis was robust when these participants were excluded, a per-protocol analysis, rather than intention to treat analysis was performed.

The 7 exacerbations requiring prolonged antibiotic therapy occurred in 6 participants. Of those, 4 completed both arms and were included in the primary ITT analysis. With these participants excluded, 8 participants who had completed both arms remained for inclusion in a per-protocol analysis group. In this group after 14 days treatment with AZLI+IV, the mean \pm SD improvement in % predicted FEV1 was $+18.5\% \pm 11.0$ in comparison to $+13.5\% \pm 8.3$ for the IV+IV treatment (mean [95% CI] treatment differences $+5.0\%$ [0.9 to 9.1], $p=0.02$).

4.3.9 Decline from baseline and subsequent recovery of lung function

To evaluate the decline from baseline on day 1 for each treatment the difference between day 1 FEV1 (% predicted) and best FEV1 (% predicted) in the preceding 6 months was calculated. The overall median day 1 drop from preceding best was -14.5% [10.5 to 20.3]. There were no significant differences between AZLI+IV and IV+IV in terms of day 1 drop from preceding best (median difference [95% CI] $+1.4\%$ [-6.0 to 17.0], $p=0.69$). Recovery of lung function (defined as achieving $>90\%$ of the best FEV1 from the preceding 6 months, as previously described [294,295], was achieved in 7/12 (58.3%) participants receiving AZLI+IV and 8/16 (50.0%) receiving IV+IV.

Figure 4.3.9: Pairwise comparison of lung function on day 1, expressed in volume (A) and as % predicted (B). Relative improvements in FEV1 (% predicted) at day 14 are presented in (C).



4.4 Discussion:

This is the first prospective study of the use of AZLI for the treatment of acute pulmonary exacerbations in people with CF and showed the combination of AZLI and intravenous colistimethate was associated with significant improvement in lung function after 14 days. Furthermore, the cross-over study design allowed a paired analysis, which confirmed the improvements after 14 days of the AZLI treatment arm were significantly greater than those seen for standard dual intravenous antibiotic therapy. In addition to the superior lung function improvements seen in the AZLI treatment arm, more participants achieved the MCID for CFQ-R Respiratory domain following the AZLI treatment arm than after standard intravenous therapy.

The superior improvements seen here for the AZLI+IV treatment equated to an extra 4.6% predicted FEV1 at day 14 and were robust when considered as absolute improvements, relative improvement or when adjusted for baseline FEV1. For both treatments, the majority of lung function improvement was achieved in the first week and relative improvements were similar between groups at day 7 suggesting the difference between treatments was established in the second week. Indeed the IV+IV group saw limited lung function improvement in the second week in keeping with previous studies of CF exacerbations, where intravenous antibiotic treatment has been found to improve FEV1 dramatically in the first week of treatment but plateau in the second week and average time to peak FEV1 is approximately 8-9 days. [296–299] A poor FEV1 response during treatment for an exacerbation is associated with failure to recover pre-exacerbation lung function and occurs in up to 25% of exacerbations treated with dual intravenous antibiotics. [148,277,299,300] In that regard, the continued improvements observed in the second week of treatment with AZLI+IV and subsequent superiority over the current standard practice of dual intravenous antibiotics may therefore be of particular clinical significance.

Inhaling antibiotics for the treatment of acute chest infections is not a novel approach given clinical trials were conducted as early as the 1980s. [247–249] Those early studies simply re-purposed intravenous preparations and administered them

via a nebuliser, a crude approach given some constituents of intravenous antibiotics are associated with lung irritation, early results were unsurprisingly mixed.

Although a large observational study in the US in 2012 reported ~10% exacerbations were treated with inhaled antibiotics in combination with intravenous antibiotics, it is not clear whether this was the addition of a new inhaled antibiotic or simply continuation of long-term inhaled therapies.[225] Outcome data for the combination of inhaled and intravenous antibiotics were not reported, however it was observed that inhaled plus oral antibiotics were superior to oral antibiotics alone. [225] Only one other prospective clinical trial of an antibiotic optimised for inhalation in the acute setting has been reported. There, tobramycin nebuliser solution was found to hold superior clinical benefits over IV tobramycin, in terms of time to next exacerbation and also renal safety outcomes. [251] The recent expansion of available inhaled antibiotics should allow further investigation of inhaled antibiotics as a treatment strategy in CF pulmonary exacerbations.

There are good reasons to expect inhaled antibiotics to be effective in the treatment of acute pulmonary exacerbations given the delivery of much greater antibiotic doses to the airways, with considerably less systemic exposure. The higher concentration of antibiotic in the lung could theoretically reduce development of antimicrobial resistance whilst also reducing the risk to the patient of drug toxicity.

Whilst the reduced systemic exposure associated with AZLI may be considered a benefit in terms of reduced toxicity, it could also be perceived as a potential limitation. For example, some exacerbations are associated with significant systemic inflammation and the reduced systemic absorption of inhaled therapies may represent insufficient treatment in those circumstances. To investigate this, serum white cell counts and CRP were compared before and after each treatment. Neither standard dual intravenous therapy nor AZLI+IV were associated with changes in white blood cell counts at day 14, a surprising finding given reductions in white blood cell counts have been reported previously following intravenous antibiotic treatment

[301,302]. However, in contrast to those previous studies where concurrent corticosteroid use was 0% and 17% respectively, in the present study nearly all participants received systemic corticosteroids. Thus, any effect on white blood cell counts may have been masked by the prolonged neutrophil survival associated with corticosteroid therapy. [303]

CRP is well validated as a predictor of treatment outcomes in CF pulmonary exacerbations, where an elevated CRP at admission is associated with reduced treatment response and shorter time until next exacerbation. [104,281,282] Here, in keeping with prior work, a raised CRP at treatment initiation was associated with poorer response to treatment. [238] This effect was similar across both treatment groups and overall reductions in CRP at day 14 were similar between the two treatment groups. Importantly, in those with raised CRP at day 1, rates of normalisation at day 14 were also similar between treatments. Taken together, there is no evidence to suggest IV+IV modulated systemic inflammation superiorly to AZLI+IV.

In the survey of UK CF clinicians set out in Chapter 2, another concern was identified regarding low systemic concentrations of inhaled antibiotics during exacerbations. This concern related to the deposition of inhaled antibiotics at the site of infection, where airways may be plugged with mucus. Little is known about the fate of inhaled antibiotics beyond the central airways, but areas of infection may be associated with increased mucus production, plugging of the airways, subsequent localised ventilation defects and thus potentially reduced penetration and antimicrobial activity for an agent delivered via the inhaled route. In that scenario, high systemic concentrations of antibiotic may be required to deliver antibiotic via the pulmonary vasculature. Computational flow dynamics have been used in attempts to model AZLI deposition in the small airways and suggested that small airways in more diseased lobes received a lower dose of AZLI than other parts of the lung, yet even those airways with lower deposition were generally predicted to have concentrations 10-fold greater than the MIC₉₀. [245] This study is unable to answer questions regarding

deposition and penetration of AZLI within the lung, but the positive effects seen on lung function and quality of life are reassuring that antibiotic deposition is adequate when given by the inhaled route.

The cross-over design used in this study allowed each participant to act as their own control and increased the statistical efficiency of the study. Cross-over studies have been used frequently in CF research over the years and the Cochrane Cystic Fibrosis and Genetic Disorders Group recently identified over 200 cross-over trials included in their reviews. [289] However, the use of this design has previously attracted controversy, mostly due to the risk of carryover effects. For example, in an early study of long-term macrolide therapy published in the Lancet it was later suggested that a carryover effect may have accounted for some of the observed treatment effect. [304] Subsequently, parallel studies have been suggested as preferable to cross-over studies where additional arguments against cross-over studies include increased length of study, treatment periods and risk of exacerbation requiring discontinuation of participation in the trial. [305] Concerns regarding washout periods and carry-through effect are largely negated in acute pulmonary exacerbations as any benefit from treatment of a previous exacerbation has, almost by definition, been lost at the time of the next exacerbation. Equally, concerns regarding discontinuations due to exacerbations are irrelevant in this setting, where exacerbations and their management are the focus of research. A further concern regarding the applicability of crossover studies to the acute exacerbation setting is the theoretical variety in exacerbation endotype. Aetiological variation between exacerbations are likely to exist but remains poorly understood and, as such, treatments remain largely empirical and standardised regardless of aetiology. The crossover design therefore remains valid. Reassuringly, day 1 clinical parameters such as drop in lung function and inflammatory were similar between exacerbations. An important advantage of the cross-over design in acute exacerbation is the possibility of paired analysis, which not only increases statistical efficiency meaning fewer participants are required, but also reduces the inter-personal variation that exists between CF exacerbations. This study confirms that cross-over studies are

feasible in the CF exacerbation setting and may serve as a useful model for future studies in this area.

There are a number of limitations to consider for this study. The study sample-size was small and conducted in a single-centre, hence the generalisability is uncertain. The cross-over design increased the statistical power sufficiently to discern the positive effect of AZLI+IV on lung function, however the study was likely underpowered to find statistical differences in the sub-group analyses, which should be considered exploratory. Future larger studies would allow more certain conclusions to be drawn in terms of differences between treatments for inflammation, quality of life and safety outcomes. Future studies will be informed by the findings in this study, both in terms of power calculations, but also in terms of feasibility and recruitment. The despite similar numbers of women and men being screened, recruitment of only one female participant in this study is an important limitation and will need to be considered in future studies to ensure generalisability of results.

In terms of inflammation, only systemic inflammation was measured and hence no assessment of the effect of AZLI+IV on airway inflammation was made. Intravenous antibiotic treatment of exacerbations effectively resolves systemic inflammation but has less impact on airway inflammation, further studies are required to examine whether an inhaled antibiotic approach could modulate airway inflammation in exacerbations. [283,306]

Participants were not blinded to treatment and hence there exists an intrinsic source of bias in that regard. Blinding of two different treatment modalities is practically challenging in this setting and would result in considerably increased treatment burden for the study participants. Fourth, allowing clinician discretion as regards the second IV antibiotic in the IV+IV arm restricts some of the conclusions that can be drawn from this data. Consideration of mandating aztreonam as the second intravenous agent in the IV+IV treatment group was given but opted against given IV

aztreonam is not used routinely as a first line anti-pseudomonal during acute exacerbations. It would thereby not serve as a relevant comparator, particularly when considering the relevance of this study to future larger studies. A pragmatic decision was therefore taken to allow clinician discretion regarding the second IV antibiotic, such that the IV+IV group was a “real-world” comparator group.

A further limitation lies in the pre-specified decision to limit study treatment to 14 days. A quarter of patients required prolongation of treatment beyond 14 days, however we did not record reasons for need of >14 days treatment and are therefore unable to evaluate possible trends in the requirement for treatment prolongation [224]

The strengths of this study lie in the successful completion of nearly 90% treatment periods, the use of a cross-over design to demonstrate the efficacy of the AZLI+IV approach and rigorous documentation of adjunctive therapies to allow comparison between groups. Importantly, the findings of this study are relevant to the CF community and addresses a number of the top ten research priorities recently established by a James Lind Alliance, namely the need to identify which antibiotic combinations are effective in exacerbations and the need to reduce negative adverse effects of antibiotics. [285] [219] Furthermore, the use of inhaled antibiotics during exacerbation has been highlighted by systematic reviews from Cochrane and others as an area in need of more evidence. [252,284]

In conclusion, this study demonstrates AZLI in combination with intravenous colistimethate, safely improves lung function and quality of life in acute pulmonary exacerbations of CF. Taken together with previous studies, these results support the notion inhaled antibiotics can represent a viable treatment approach for some acute pulmonary exacerbations and larger studies are warranted to confirm these findings and define how inhaled antibiotics can be optimally incorporated into clinical practice.

Chapter 5: Changes in Bacterial Load and Aztreonam Resistance in the AZTEC-CF study

5.1 Introduction

The AZTEC-CF study, described in detail in Chapter 3 & 4, tested the use of AZLI+IV against IV+IV for the treatment of acute pulmonary exacerbations of CF. The aims of this Chapter were firstly, to compare the effect of each treatment on bacterial load and aztreonam resistance, and secondly, to explore how any changes were associated with clinical outcomes.

5.2 Methods

5.2.1 AZTEC-CF Study

The AZTEC-CF study methods are set out in detail in Chapter 3. Briefly, AZLI plus intravenous colistimethate (AZLI+IV) was tested against standard care dual intravenous antibiotics (IV+IV) for the treatment of acute pulmonary exacerbations of CF. Each treatment course was 14 days with sputum sampling performed on Day 1, prior to first antibiotic dose, and Day 14, after last dose of antibiotic.

5.2.2 Sample collection

Sputum samples were collected in sterile containers and immediately refrigerated at 4°C for up to 24 h before being flash frozen in liquid nitrogen and stored at -80°C. Once defrosted, an equal volume of diethiothreitol 1.4% w/v (Sputasol, Oxoid, UK) was added before 30 min incubation at 37°C on a shaker at 300 rpm. Where sputum samples were of low volume, they were prioritised for culture independent analyses.

5.2.3 Media preparation

Four different media were prepared to facilitate calculation of total bacterial load, total *P. aeruginosa* load, aztreonam-resistant bacterial load and aztreonam-resistant *P. aeruginosa* load respectively. For total bacterial load a non-selective nutrient rich agar (Luria-Bertani Agar, Oxoid, UK) and for total *P. aeruginosa* load a *Pseudomonas*-selective agar (*Pseudomonas* (CN) Selective Agar, Oxoid, UK) were prepared. To

evaluate the respective aztreonam-resistant loads, 16 µg/ml aztreonam, the reference minimum inhibitory breakpoint (MIC), dissolved in dimethyl sulfoxide was added to each media. [307] NB: It is important to note that where “total bacterial load” is referred to herein, it represents the total culturable load in standard culture conditions. Extensive culture including anaerobic cultures were not performed.

5.2.4 Dilution series

A dilution series for each sputum sample was created in sterile phosphate buffered saline (PBS) at concentrations down to 10^{-11} by the addition of 20 µl of sputum to 180 µl of sterile PBS, vortexing thoroughly, then adding 20 µl of the vortexed mixture to 180 µl of PBS into a second tube and sequentially repeating that process.

5.2.5 Quantitative Culture

10 µl of each dilution was plated onto four media in triplicate. After 48 h incubation at 37°C, colonies were counted at the dilution where 10-100 colonies were visible. Colony forming units per millilitre (CFU/ml) were subsequently calculated as follows:

$$CFU/ml = \text{No. of colonies counted in plate segment} \times \text{dilution factor} \times 2 \times 100$$

5.2.6 Quantitative polymerase chain reaction

Determination of sputum bacterial load by quantitative polymerase chain reaction (qPCR) was undertaken by targeted amplification of the 16S rRNA gene as previously described. [308] Standards were generated by conventional PCR using the Promega GoTaq kit (Promega, UK) and the following cycling conditions: 2 min 95°C, 30 cycles of 30 secs 95°C, 1 min 60°C, 1 min 72°C, and a final extension step of 72°C for 5 mins. A dilution series of the amplified DNA of known copy number was made and five dilutions analysed in order to generate a standard curve.

The Qiagen Rotorgene SybrGreen qPCR kit (Qiagen, UK) was used for qPCR. The 25 µl reaction mix included: 12.5 µl SYBR green PCR master mix, 1 µl of forward (5'-

TCCTACGGGAGGCAGCAGT-3') and reverse (5' GGACTACCAGGGTATCTAATCCTGTT-3') primer, 8.5 µl water and 2 µl DNA. Controls of RNase and DNase free water were also analysed in duplication. qPCR was carried out using a Qiagen rotor-gene, using a three step with melt method. Results were analysed using the two standard curves method.

5.2.7 Electronic health record

Metadata were collected from the local electronic health record (EHR) at Liverpool Heart and Chest Hospital with a focus on anthropomorphic data and prior antibiotic usage. The EHR (Allscripts®, Manchester, UK) has been in place since 2013 and routinely collects all intravenous antibiotic prescriptions made at LHCH into a structured database, allowing retrieval of total and individual intravenous antibiotic use from 2013 to 2017.

5.2.8 Statistical analysis

Statistical analyses were conducted in RStudio (v1.0.136, R Studio Inc). All CFU/ml data was log-transformed. Between-group comparisons were made using Mann-Whitney or Wilcoxon's signed-rank tests for unpaired and paired data respectively. Associations between continuous variables were tested using Pearson's correlation coefficient. Unadjusted *p*-values are presented throughout and a significance level of <0.05 was considered significant.

5.3 Results

5.3.1 Study population

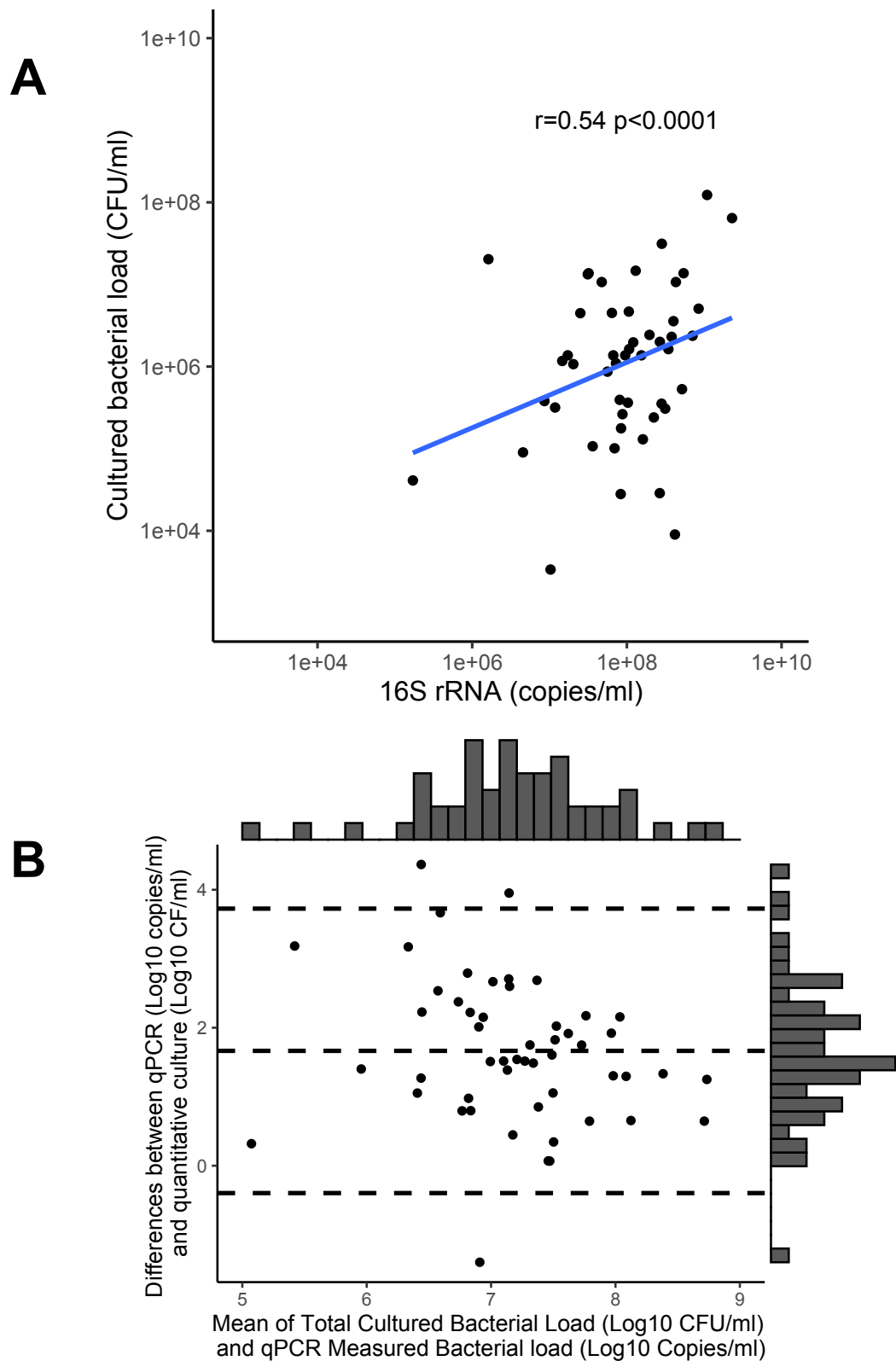
Participants from the AZTEC-CF study with available pre and post sputum samples for at least one treatment arm were included. All participants in the AZTEC-CF study were AZLI naïve. The IV+IV arm was completed by 16 participants, of whom 13/16 (88%) had paired sputum samples available for culture. The AZLI+IV arm was completed by 12 participants of whom 11/12 (92%) had suitable paired samples available. The excluded participant provided two samples, but one was consistently overgrown with fungus, making interpretation of bacterial loads and AMR impossible and was thus excluded from analysis.

On day 1, median [IQR] cultured total bacterial load was 6.4 log₁₀CFU/ml [5.8 to 7.0] and total bacterial load detected by qPCR was 8.1 log₁₀ 16S copies/ml [7.7 to 8.6]. *P. aeruginosa* was detected in culture in all day 1 samples with a median [IQR] cultured load of 4.5 log₁₀CFU/ml [3.2 to 4.5]. On day 14, *P. aeruginosa* was not cultured in 2/11 (18.2%) and 1/13 (7.7%) for AZLI+IV and IV+IV respectively.

5.3.2 Quantitative PCR correlates with cultured bacterial load

Cultured bacterial load was consistently lower (median [IQR] difference -1.6 log₁₀ counts [-1.9 to -1.3], $p < 0.0001$) than that measured by qPCR, but there was a moderate correlation between both measures ($r = 0.54$, $p < 0.001$), see Figure 5.3.1. A Bland-Altman plot was drawn to compare agreement between the two measurement modalities and showed the difference between the two measurements was generally consistent across the range of observed bacterial loads, see Figure 5.3.1.

Figure 5.3.1: Relationship between total bacterial load as measured by qPCR and quantitative culture (A). Bland-Altman plot (with mean and 95% CI represented by dotted lines) and marginal histogram demonstrating the degree of agreement and distribution between bacterial load measured by qPCR and quantitative culture (B).



5.3.3 Changes in sputum bacterial load do not differ between treatments

Changes in total bacterial and *P. aeruginosa* sputum load are presented in Table 5.3.1. Overall, neither culturable total bacterial load nor *P. aeruginosa* load had significantly changed following either treatment ($p=0.69-0.99$); see Figure 5.3.1 & Figure 5.3.2. On an individual level, after 14 days AZLI+IV 5/11 (45%) participants had reductions ≥ 1 Log in sputum *P. aeruginosa* load in comparison to 3/13 (23%) in the IV+IV group (Fisher's exact $p=0.36$). There was wide variation in changes ranging from $-2.0 \log_{10}\text{CFU/ml}$ to $+2.6 \log_{10}\text{CFU/ml}$ for total bacterial load, and $-3.3 \log_{10}\text{CFU/ml}$ to $+3.6 \log_{10}\text{CFU/ml}$ for *P. aeruginosa*, see Figure 5.3.3.

A similar pattern was seen when bacterial load was measured by qPCR, for example no change in total 16S copies was observed after 14 days treatment with AZLI+IV (median [IQR] day 1 vs. day 14: $8.1 \log_{10}\text{CFU/ml}$ [7.5 to 8.6] vs. $7.9 \log_{10}\text{CFU/ml}$ [7.2 to 8.0], $p=0.43$) or IV+IV (median [IQR] day 1 vs. day 14: $8.1 \log_{10}\text{CFU/ml}$ [7.9 to 8.6] vs. $8.2 \log_{10}\text{CFU/ml}$ [7.5 to 8.5], $p=0.19$), and individual differences ranged from -2.5 to $+0.9 \log_{10}\text{CFU/ml}$.

Table 5.3.1: Sputum bacterial load on day 1 and 14 for each treatment. Data are presented as median [IQR] Log10 CFU/ml for quantitative culture or Log10 copies/ml for qPCR

Paired analysis by Wilcoxon Signed-rank test, unadjusted *p*-values

	AZLI + IV		IV+IV	
	Day 1	Day 14	Day 1	Day 14
<u>Quantitative culture</u>				
<u>n</u>	11	11	13	13
Total Bacteria	5.8 [5.4 to 7.3]	5.9 [5.3 to 7.4]	6.6 [6.3 to 7.0]	6.4 [5.7 to 7.0]
Aztreonam-resistant	5.3 [3.6 to 6.0]	5.9 [5.0 to 7.3]	6.4 [6.0 to 6.6]	6.0 [5.6 to 6.6]
Total <i>P. aeruginosa</i>	3.4 [3.1 to 4.7]	3.3 [1.8 to 4.3]	4.9 [3.3 to 5.5]	5.0 [3.2 to 5.6]
Aztreonam-resistant	0.1 [0 to 3.6]	1.8 [0 to 4.7]	2.1 [0 to 3.8]	4.0 [0 to 5.0]
<u>qPCR</u>				
<u>n</u>	12	12	16	16
Total 16S copies	8.1 [7.5 to 8.6]	7.9 [7.2 to 8.0]	8.07 [7.9 to 8.6]	8.2 [7.5 to 8.5]
				0.19

Figure 5.3.2: Comparison of cultured bacterial loads pre and post treatment with AZLI+IV (orange): Total bacterial load (A) and *P. aeruginosa* load (C) and IV+IV (blue): Total bacterial load (B) and *P. aeruginosa* load (D)

Paired analysis by Wilcoxon Signed-rank test

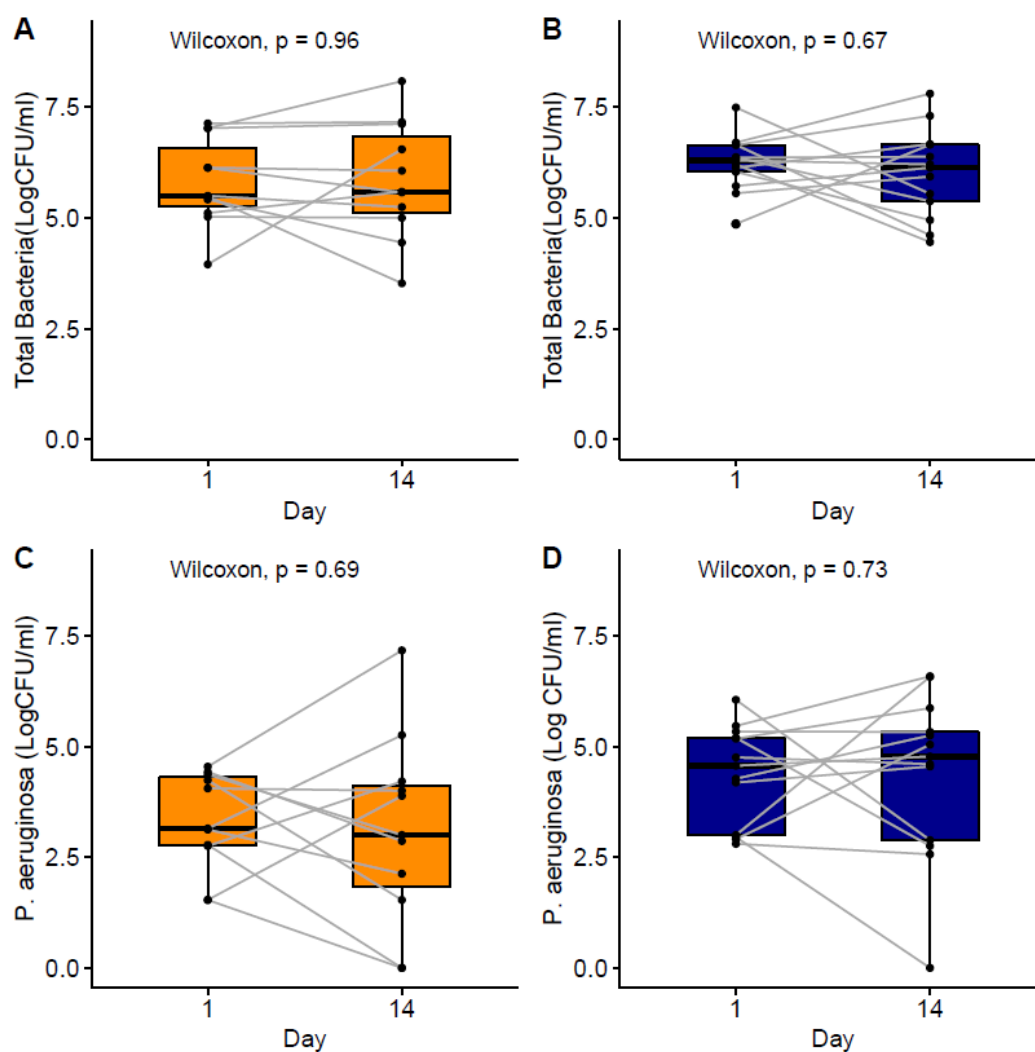
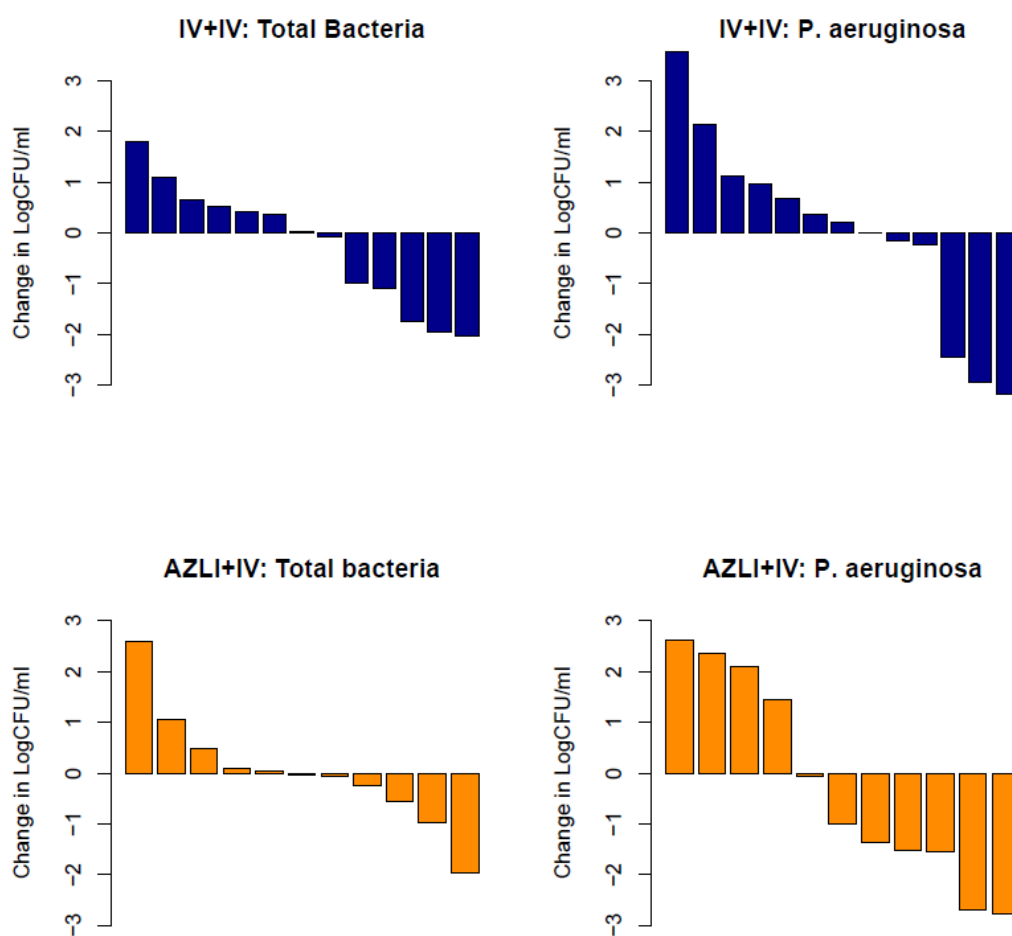


Figure 5.3.3: Waterfall plot of individual changes in cultured total bacterial load and *P. aeruginosa* load for AZLI+IV (orange) and IV+IV (blue)



5.3.4 Changes in bacterial load are associated with quality of life, but not lung function.

Next the relationship between changes in bacterial load and clinical outcomes was examined. On day 1 there were no relationships observed for bacterial load with either lung function or quality of life (CFQ-R Respiratory Domain), see Figure 5.3.4 and Figure 5.3.5. After 14 days treatment, no significant relationship was found between change in bacterial load and lung function ($r = 0.24$, $p = 0.27$) or *P. aeruginosa* load and lung function ($r = -0.33$, $p = 0.14$). However significant moderate inverse relationships were observed between respiratory symptoms on day 14, as measured by the CFQ-R Respiratory domain, and changes in both total bacterial load ($r = -0.41$, $p = 0.05$) and *P. aeruginosa* load ($r = -0.49$, $p = 0.01$), see Figure 5.3.4 and Figure 5.3.5.

5.3.5 Aztreonam resistance is prevalent regardless of previous aztreonam exposure

At study entry i.e. first day of first admission, aztreonam-resistant *P. aeruginosa* CFUs were observed in 8/16 (50%) study participants. EHR interrogation confirmed no record of previous aztreonam exposure in any participant. We therefore tested the hypothesis that those with aztreonam resistance would be different in terms of clinical characteristics and prior antibiotic exposure and found that although patients harbouring aztreonam-resistant isolates were similar in terms of age, lung function and LES status, aztreonam resistance was associated with increased intravenous meropenem use in years prior to study entry (median days [IQR] 43.5 days [13.8 to 56.8] vs. 9 [0 to 18], $p = 0.05$), see Table 5.3.2. Baseline aztreonam resistance did not appear to be associated with poorer clinical outcomes for either treatment.

Figure 5.3.4: Pearson's correlation coefficients between sputum bacterial load and lung function

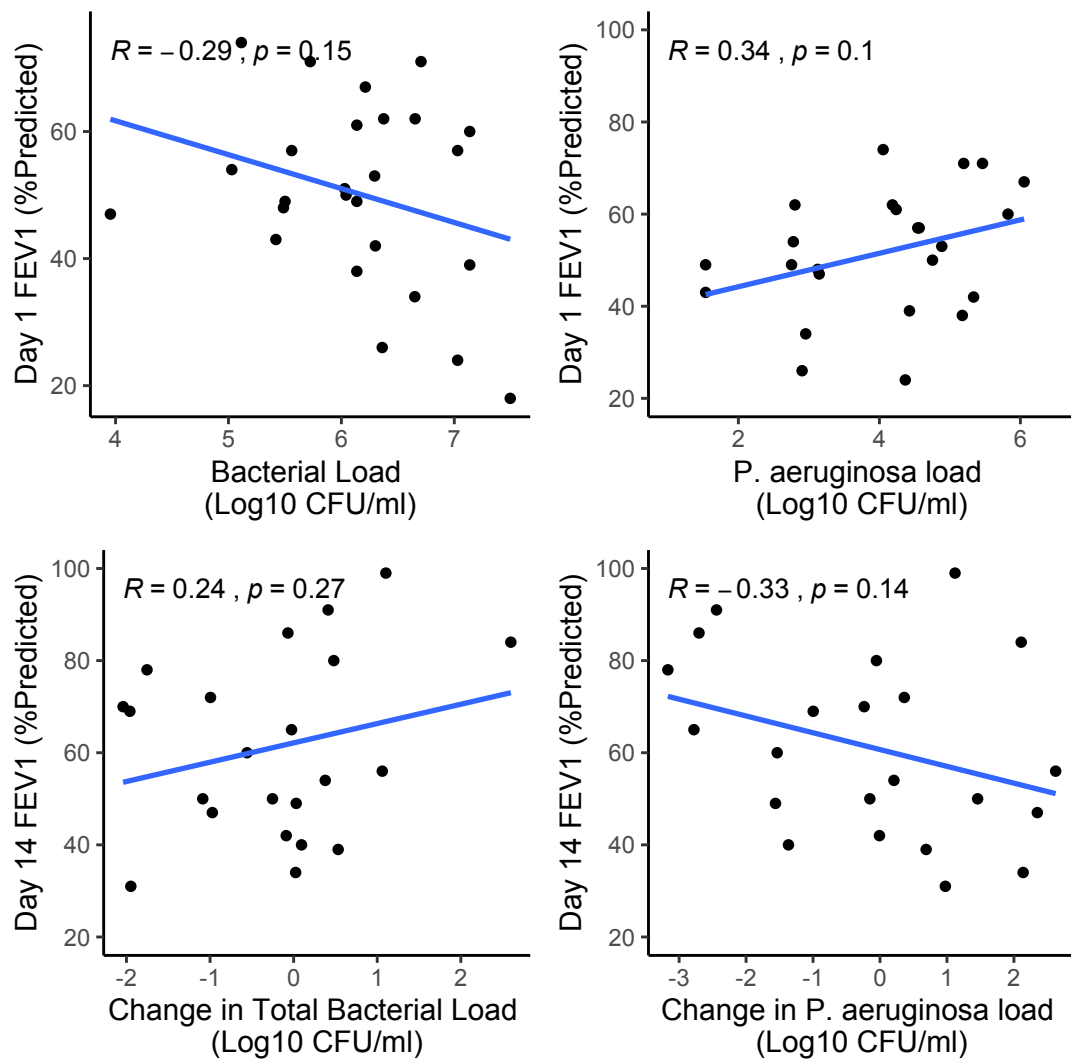
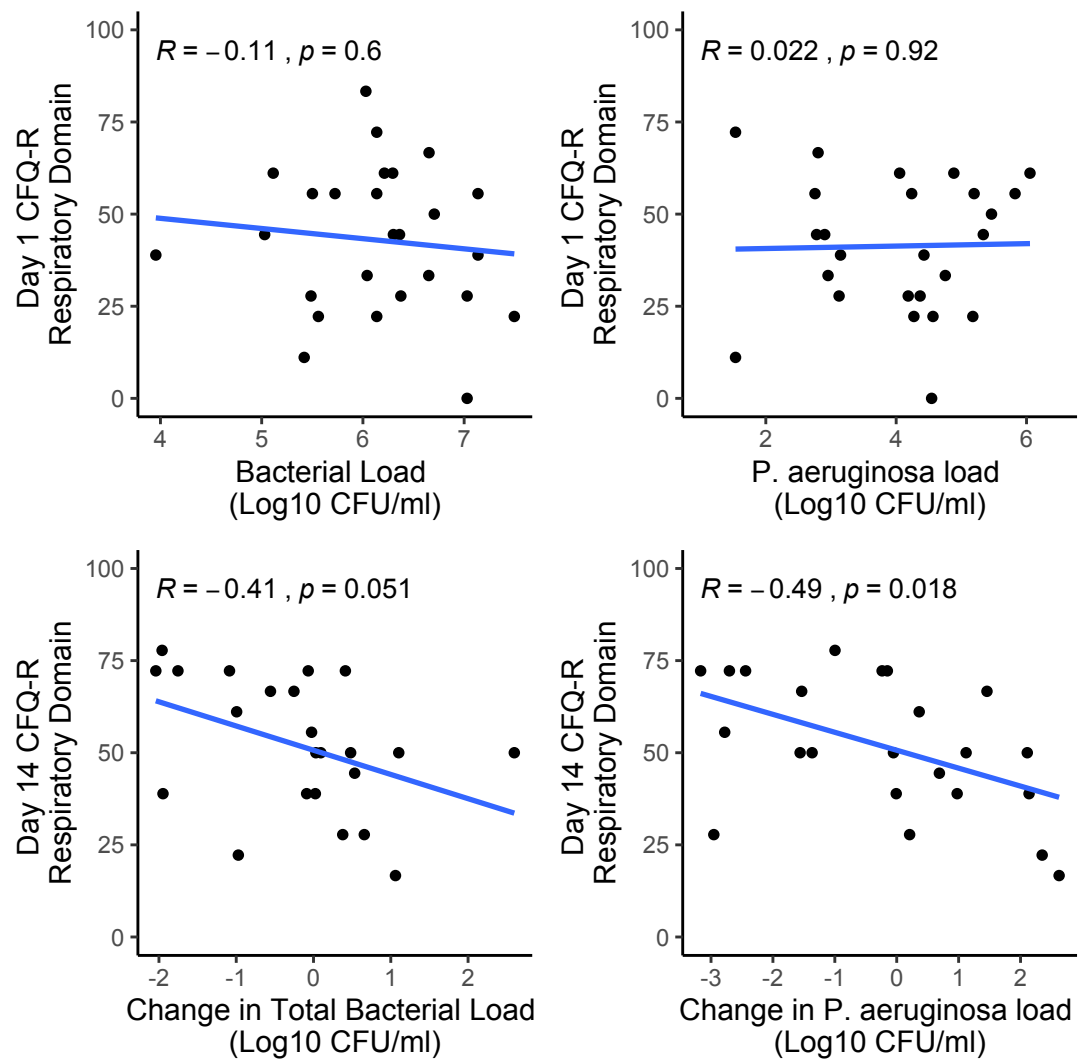


Figure 5.3.5: Pearson's correlation coefficient between sputum bacterial load and quality of life



5.3.6 Aztreonam resistance increased following treatment with IV+IV but not AZLI+IV

At initiation of AZLI+IV, 5/11 participants (45%) harboured aztreonam-resistant *P. aeruginosa* and after 14 days treatment there was no difference in aztreonam-resistant pseudomonal load, see Table 5.3.1 & Figure 5.3.6. At initiation of the IV+IV treatment, 8/13 (62%) participants harboured aztreonam-resistant isolates and after 14 days treatment aztreonam-resistant increased (median difference [95% confidence interval] +0.9 log₁₀ CFU/ml [0.3 to 1.9], p=0.01). The increased aztreonam resistance seen after 14 days treatment with IV+IV appeared to be driven exclusively by those 8 participants with resistance at baseline given the 5 participants without aztreonam resistance at baseline were all still susceptible at day 14, whereas the 8 participants with aztreonam resistance at baseline all saw increased resistance at day 14 (median difference [IQR] +1.1 log₁₀ CFU/ml [+1.0 to +1.7]), see Figure 5.3.6D.

Table 5.3.2: Comparison of clinical characteristics and prior antibiotic by aztreonam resistance at study entry.

Data are presented as median [IQR] or count (%). Statistical comparison by Mann-Whitney U test or Fisher's Exact test, unadjusted *p*-values.

	Aztreonam susceptible	Aztreonam resistant	p
n	8	8	
Age	30.4 [22.8, 31.7]	28.34 [25.4, 30.4]	0.60
Male	7 (87.5)	8 (100.0)	0.99
DF508 homozygous	3 (37.5)	8 (100.0)	0.03
BMI	18.9 [17.8, 23.8]	22.3 [21.6, 23.3]	0.25
FEV1, % predicted	46.0 [38.8, 62.0]	60.0 [54.8, 66.3]	0.19
<i>P. aeruginosa</i>	8 (100.0)	8 (100.0)	0.99
LES	4 (50.0)	6 (75.0)	0.61
<i>S. aureus</i>	2 (25.0)	2 (25.0)	0.99
Long-term antibiotics			
Oral macrolide	8 (100.0)	7 (87.5)	0.99
Colistimethate	8 (100.0)	6 (75.0)	0.45
Tobramycin	4 (50.0)	2 (25.0)	0.61
Annual IV days	28.0 [10.5, 45.8]	23.0 [13.8, 37.5]	0.96
Prior IV exposure 2013-2017, days			
Fosfomycin	0.0 [0.00, 3.3]	8.0 [0.0, 27.8]	0.13
Tobramycin	18.0 [3.8, 22.8]	18.5 [0.0, 81.8]	0.63
Colistimethate	43.0 [17.8, 57.0]	54.5 [16.8, 61.5]	0.75
Meropenem	9.0 [0.0, 18.0]	43.5 [13.8, 56.8]	0.05
Ceftazidime	33.0 [24.0, 96.5]	28.0 [7.8, 46.8]	0.29
Co-trimoxazole	0.0 [0.0, 0.0]	0.0 [0.0, 0.0]	
Aztreonam	0.0 [0.0, 0.0]	0.0 [0.0, 0.0]	
Piperacillin/Tazobactam	0.0 [0.0, 16.3]	6.0 [0.0, 22.8]	0.60
Teicoplanin	0.0 [0.0, 0.0]	0.0 [0.0, 0.0]	0.93
Temocillin	0.0 [0.0, 0.0]	0.0 [0.0, 0.0]	
Total	165.0 [63.3, 228.3]	134.0 [94.3, 332.5]	0.75

BMI=Body Mass Index; FEV1=Forced expiratory volume in 1 second

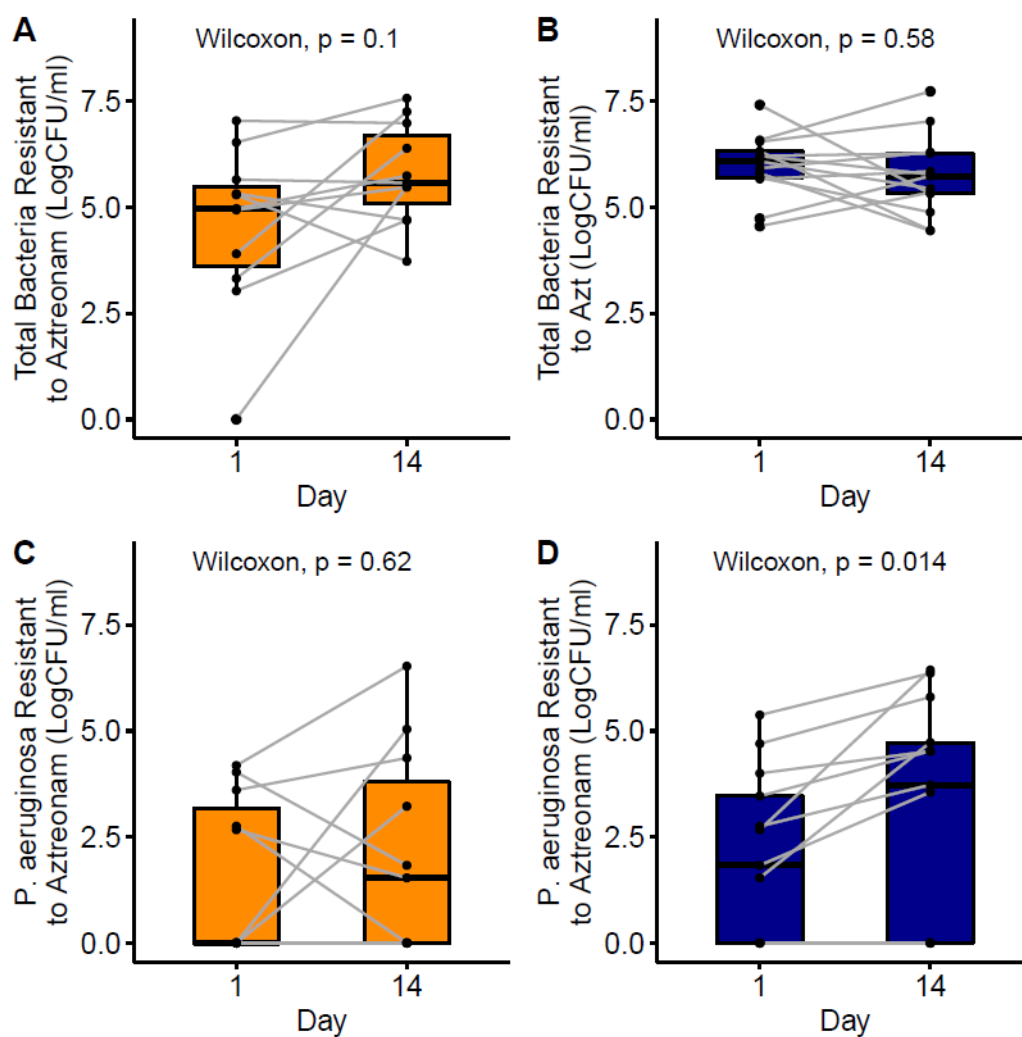
Table 5.3.3: Comparison of key clinical outcomes in participants with and without baseline aztreonam resistance.

Data are presented as median change with comparison by Wilcoxon Signed-rank test

	Aztreonam Resistance at baseline		
	Yes	No	<i>p</i>
<u>AZLI+IV</u>			
n	5	6	
Change after 14 days			
FEV1, % <i>predicted</i>	+10.5	+11	0.84
CFQ-R Respiratory domain	+13.9	+11.1	0.76
White cell count	+1.7	+0.1	0.33
C-reactive protein	-3.5	0.0	0.64
Total Bacteria	+0.1	-0.6	0.13
<i>P. aeruginosa</i>	-1.5	+1.5	0.25
<u>IV+IV</u>			
n	8	5	
Change after 14 days			
FEV1, % <i>predicted</i>	+9	+8	0.67
CFQ-R Respiratory domain	+16.6	+5.6	0.50
White cell count	-0.7	-0.9	0.99
C-reactive protein	-24	-21	0.99
Total Bacteria	-1.1	+0.4	0.23
<i>P. aeruginosa</i>	-0.1	+0.2	0.88

Figure 5.3.6: Comparison of aztreonam-resistant sputum bacterial load pre and post treatment with AZLI+IV (orange): Total aztreonam-resistant load (A) and aztreonam-resistant *P. aeruginosa* load (C) and IV+IV (blue): Total aztreonam-resistant load (B) and aztreonam-resistant *P. aeruginosa* load (D)

Comparison by Wilcoxon Signed-rank test



5.4 Discussion

This study compared the impact of AZLI+IV and IV+IV on sputum-culture bacterial load and aztreonam resistance. Overall, there was considerable variability in treatment responses and neither treatment consistently reduced total bacteria or *P. aeruginosa* counts. On an individual level, large reductions were observed for some participants and the degree of reduction in *P. aeruginosa* load was found to be significantly associated with improved subjective response to treatment. After 14 days treatment, no overall differences in aztreonam resistance were found for AZLI+IV, however a surprising finding was increased aztreonam resistance after IV+IV treatment, despite those treatment regimens not including aztreonam.

The lack of overall change in total bacterial or *P. aeruginosa* load seen here is at odds with early studies of CF exacerbations. For example, in 1988 Smith *et al.* reported hospitalisation and treatment with intravenous antibiotics was associated with reductions from 7.8 to 4.6 locus/ml. [309] Similarly in 1990, Regelman *et al.* found antibiotic treatment of exacerbations was associated with >2-Log reductions in *P. aeruginosa* CFUs. [310] However, these studies were performed prior to the routine use of long-term inhaled antibiotics targeted towards *P. aeruginosa* and the day 1 sputum loads reported in those early studies (~8 LogCFU/ml) are greater than the baseline *P. aeruginosa* densities seen here. The results seen here are similar to a recent study which found IV antibiotic treatment of acute pulmonary exacerbations resulted in reduced *P. aeruginosa* counts only 60% of the time, and confirm the findings of other studies from the last decade where no consistent changes in sputum bacterial load were observed during treatment of exacerbations. [149,311–314]

Although neither treatment was associated with overall changes in *P. aeruginosa* load, changes were evident on an individual basis where some participants had substantial reductions in sputum counts. A significant relationship between changes in bacterial load and quality of life was observed, such that greater reductions in bacterial load were associated with improved patient-reported quality of life. In

contrast, there was no relationship between changes in sputum load and lung function, in keeping with prior work where lung function and quality of life metrics have previously been found to be incongruent in the acute exacerbation setting. [277,315,316] It is plausible that lung function and patient reported quality of life outcomes measure different aspects of disease. For example, FEV1 (the primary outcome measure in the AZTEC-CF study) is notoriously insensitive to changes in small airways, and a reduced bacterial load could theoretically accompany unplugging of obstructed small airways, resulting in improved symptoms but no change in FEV1. [317]

Surprisingly, aztreonam-resistant *P. aeruginosa* was present in half of participants despite all being AZLI-naïve. Interrogation of the local electronic health record confirmed no prescription of intravenous aztreonam in the previous five years although historical exposure at this centre, or in paediatrics cannot be entirely ruled out. Interestingly, those with evidence of aztreonam resistance at baseline had significantly greater resistance after fourteen days of IV+IV despite those regimens consisting of colistimethate with meropenem or ceftazidime rather than aztreonam, see Table 4.3.2. These findings may be explained by the shared mechanisms of resistance between aztreonam and other commonly used anti-pseudomonals. For example, meropenem and ceftazidime are both also known substrates for β -lactamases and the MexAB-OprM efflux system, which are antimicrobial resistance mechanisms naturally encoded within the *P. aeruginosa* chromosome, but not always expressed at high levels. [318–320] Antibiotic exposure can select for populations with mutations in key regulatory genes where overexpression of β -lactamases increase the ability of *P. aeruginosa* to enzymatically inactivate antibiotics and de-repression of the MexAB-OprM efflux system allows removal of the antibiotic from the cell entirely. [75]

Increased prior exposure to meropenem was noted in participants with baseline aztreonam resistance, in keeping with previous work which identified meropenem as a key driver of multi-drug resistance in clinical *P. aeruginosa* strains. [321]

Collateral resistance refers to an antimicrobial agent inducing resistance to another agent and we hypothesise that historical up-regulation of resistance mechanisms, in response to exposure to other anti-pseudomonals such as meropenem, resulted in collateral baseline resistance to aztreonam despite no aztreonam exposure.

Overall, no increased aztreonam resistance after treatment with AZLI+IV was observed and this finding is relevant to the clinical utility of AZLI in the acute setting where it remains an important chronic suppressive agent against *P. aeruginosa*. Although the relationship between susceptibility testing and clinical response is not well established in CF, had AZLI+IV been associated with increased aztreonam resistance clinicians may be more wary of adopting AZLI+IV as a treatment in the acute setting for fear of reducing efficacy when needed in the chronic setting in the future.

Our finding of increased aztreonam-resistant pseudomonal load in the IV+IV group, but not AZLI+IV group is in keeping with findings from studies in ventilator-acquired pneumonia where nebulised antibiotic delivery has been associated with reduced antimicrobial resistance compared to intravenous antibiotics. [267,322] As described previously, aztreonam is a substrate for similar resistance mechanisms to other anti-pseudomonal antibiotics, which may explain the increased resistance seen in the IV+IV group, however this does not explain the lack of a similar pattern in the AZLI+IV group. It is important to recognise increases in MIC below the reference breakpoint cannot be ruled out but an explanation for these findings may lie in the high dose of antibiotic delivered to the lung with nebulised treatments (up to 100-fold greater with AZLI than IV aztreonam). [206] Such high doses may exceed the mutant prevention concentration (MPC) more often than systemically delivered antibiotics. [323] Alternatively, evolutionary theory suggests higher doses of antibiotic may lead to mutations that result in higher resistance, but also associated higher fitness costs, which if too high will not be selected for and resistance will not subsequently increase. [324]

There are a number of limitations to consider for this study. First, although 16 participants were recruited to the AZTEC-CF study, quantitative culture of paired samples was only possible for 11 and 13 participants for the AZLI+IV and IV+IV treatments respectively. The AZTEC-CF study was not powered toward microbiological end-points and hence findings here should be considered hypothesis generating. For example, although no overall increased resistance to aztreonam was seen with AZLI+IV, there were considerable changes on an individual level and a better powered study would allow firmer conclusions on the reproducibility and relevance of these findings. Second, sputum was sampled at beginning and end of treatment only and cannot exclude a transient treatment effect as reported previously by Deschaght *et al.*, where *P. aeruginosa* counts had reduced after 8 days treatment with intravenous antibiotics, but by day 15 had increased back towards day 1 values. [325] Third, given aztreonam was the investigational agent of interest in the AZTEC-CF study, only aztreonam resistance was measured and therefore no assessment on whether AZLI+IV also induced collateral resistance could be made. Fourth, aztreonam resistance was assessed based upon the reference MIC breakpoint rather than determining individual MICs for each sputum sample. As a consequence, it is not known whether individual MICs increased towards the reference breakpoint during treatment.

5.5 Conclusion

Neither IV+IV nor AZLI+IV consistently reduced sputum bacterial load but where bacterial load was reduced, it was associated with improved respiratory symptoms. Overall, AZLI+IV was not associated with increased aztreonam resistance, whereas the IV+IV group did see increased aztreonam resistance, perhaps suggestive of collateral mechanisms warranting further investigation.

Chapter 6: Lung Microbiota Dynamics during the AZTEC-CF Study

6.1 Introduction

The premise and evidence base for culture-independent identification of microbial communities in the lungs of people with cystic fibrosis (CF) are set out in Chapter 1. As discussed therein, the CF lung microbiota has been extensively investigated in cross-sectional and longitudinal studies yet relatively little work has been performed in an interventional trial setting.[153,326–328] Instead, studies to date have been largely limited to comparing changes in the CF lung microbiota across different clinical states e.g. stability and exacerbation, rather than comparing treatment effects.

This chapter explores the lung microbiota of AZTEC-CF study participants with attention paid to how an individual's clinical characteristics relate to the bacterial community found within their lungs, whether each study treatment exerted different effects on the microbiota and how those changes relate to clinical outcomes.

6.2 Methods

6.2.1 AZTEC-CF Study

The AZTEC-CF study methods are set out in detail in chapter 3. Briefly, AZLI plus intravenous colistimethate (AZLI+IV) was tested against standard care dual intravenous antibiotics (IV+IV) for the treatment of acute pulmonary exacerbations of CF in a cross-over study. Each treatment course was 14 days with sputum sampling performed on Day 1, prior to first antibiotic dose, and Day 14, after last dose of antibiotic. After collection, sputum samples were immediately refrigerated at 4°C before flash freezing in liquid nitrogen and stored at -80°C within 24 h.

6.2.2 DNA extraction

For DNA extraction, samples were defrosted and extractions undertaken in batches. Freeze-time was standardised such that each participant's pre-treatment and post treatment samples were defrosted in the same batch. Once defrosted, an equal volume of diethiothreitol 1.4% w/v (Sputasol, Oxoid, UK) was added before 30 min incubation at 37°C on a shaker at 300 rpm. DNA extraction was performed using the ZymoBIOMICS™ DNA Miniprep Kit (ZymoBIOMICS, USA) in conjunction with the manufacturers protocol. This method consists of the addition of 200µl sputum to tubes containing ultra-high-density beads and lysis solution. Mechanical cell lysis was then performed by bead beating in a high-speed cell disrupter (TissueLyser II Qiagen, Manchester, UK) for 5 minutes at 25 Hz. Centrifugation allowed removal of lysate which then underwent washing and purification stages in spin-columns (ZymoBIOMICS, USA). Finally, PCR inhibitors e.g. polyphenolics, humic/fulvic acid and melanin were removed before the DNA elution in 100µl of DNase/RNase free water. Eluted DNA was stored at -80°C until sequencing.

6.3 Control samples

A number of control samples were included in the study design:

1. **Negative kit control:** A "blank" DNA extraction, where 200 µl of Invitrogen™ DEPC- Treated Water (ThermoFisher, UK) was used in place of sputum to allow assessment and identification of potential kit/reagent contamination.
2. **Negative sequencing control:** A "blank" sample, 1µl of PCR grade DNA-free water, was included alongside study samples at the PCR stage to allow assessment of de-novo contamination or cross-contamination introduced during amplification sequencing.
3. **Positive kit control:** 75 µl of a mock community (ZymoBIOMICS™ Microbial Community Standard) underwent DNA extraction and sequencing alongside all other study samples. A mock community of known composition acts as a well-defined input to the DNA extraction and sequencing process.

Comparing sequencing output to the known input allows assessment of bias and contamination associated with the extraction process.

4. **Positive sequencing control:** A defined mixture of genomic DNA isolated from pure cultures (ZymoBIOMICS™ Microbial Community DNA Standard) was included at the PCR stage to allow assessment of bias introduced by the amplification and sequencing process.

6.3.1 DNA quantification

To confirm the presence and purity of extracted DNA, eluted samples underwent quantification by microvolume spectrophotometry (NanoDrop 1000, Thermo Scientific) and fluorometry (Qubit 4, Invitrogen). Quantitative polymerase chain reaction (qPCR) was performed as set out in Chapter 5.

6.3.2 16S rRNA gene sequencing

Amplification and sequencing of the V4 hypervariable region of 16S followed a standardised and optimised protocol at NU-OMICS sequencing facility. The protocol is set out in depth by Kozich *et al.* [329] Briefly, 1µl of template DNA was added to 17µl of AccuPrime™ Pfx Supermix in a 96 well plate and 2µl of the index primers (515 (GTGCCAGCMGCCGCGGTAA) and 806 (GGACTACHVGGGTWTCTAAT). After PCR, library clean-up was performed with Invitrogen SequalPrep Plate Normalisation kit. Samples from all plates were pooled and quantified using a KAPA Biosystems Q-PCR kit. The library was sequenced in the Illumina MiSeq System (Illumina Inc., USA), including 5.0% PhiX as internal control.

6.3.3 Amplicon bioinformatics

The QIIME2 pipeline was used for quality control, sequence inference and taxonomical assignment. [330] First, low-quality reads were removed, and reads were trimmed to a 250bp length. Next, unique sequences and their read counts were computed using a parameterised model of substitution errors to distinguish sequencing errors from real biological variation. Chimeras (artefact sequences incorrectly joined in the PCR process) were removed and the remaining unique amplicon sequence variants (ASVs) were taxonomically assigned using the

Greengenes 16S rRNA database version 13.5.[331] ASVs are annotated throughout with the highest taxonomic rank achieved, for example ASVs resolved to the genus level are preceded by “g_” and those resolved to species level are preceded by “s_”.

6.3.4 Data, samples and statistical analyses

ASV table and taxonomic assignment were exported for analyses performed in RStudio (Version 1.0.136 – © 2009-2016 RStudio, Inc). Sequencing outputs were combined with meta-data and initial inspection of data was conducted in the “phyloseq” package.[332] Other subsequent packages used for analysis included “vegan”, “factoExtra” and “microbiome”. [333–335] Plots were created within “ggplot2” or “ggpubr” packages.[336,337]

To avoid repeated measures bias, only each participant’s first study sample was used (n=16) for assessments of the relationship between clinical characteristics and the lung microbiota. For between-treatment comparisons, only those participants who provided valid study samples for day 1 and 14 of each treatment (n=10, 40 samples in total) were included for analysis.

Data is presented throughout as median [IQR] or where specified for paired differences, median [95% confidence interval]. Between-treatment comparisons for continuous data were made using a paired Wilcoxon test and comparisons across study period were made using a Kruskal-Wallis test. Correlations were tested using Pearson’s Correlation Coefficient. Where multiple comparisons were being made simultaneously, e.g. associations between the microbiota and clinical characteristics, a Bonferroni correction was applied.

6.3.5 Microbiota analyses

6.3.5.1 Alpha diversity

Alpha diversity is an ecological measure of community structure. The simplest measure of alpha diversity is “richness” i.e. the observed number of different species within a sample. Other frequently used measures include the Shannon Diversity Index (also known as the Shannon-Wiener or Shannon-Weaver index) which takes into account the number of species but also their relative proportion within the community. [338] A sample made up of only a few species with an uneven distribution of abundance would yield a low Shannon Diversity Index, whereas a sample with many species, all of equal abundance would achieve a high Shannon Diversity Index. Given sample Richness and Shannon’s index are prone to bias from sampling depth, Fisher’s alpha index, which describes the relationship between the number of species and abundance of that species in a logarithmic series model, was also included. Fisher’s index is less prone to bias from sequencing depth, and is robust in the face of incomplete sampling. [339]

6.3.5.2 Core and satellite microbiota partitioning

Partitioning CF microbiota into “core” and “satellite” taxa has previously been shown to be important for revealing changes that would be neglected without such a distinction. [340] Challenges in identifying a “core” microbiota from sequencing data include many zeros in datasets and large variance in distribution patterns with certain samples dominated by particular taxa which may be completely absent in other samples. Previously, sequencing datasets have been arbitrarily filtered to remove low abundance taxa, however novel methods such as the Prevalence Interval for Microbiome (PIME) tool can be used to partition a “core” microbiota in a standardised manner. [341] PIME improves “core” partitioning by using a Random Forests analysis to determine the level of prevalence that provides the best model to predict differences in the communities while still including as many taxa as possible in the analysis. Using the PIME package, a prevalence interval of 60% was identified as an optimal threshold. Taxa prevalent in >60% of study participants were

therefore included in the “core” microbiota for Beta diversity and individual taxa analyses.

6.3.5.3 Beta Diversity

Beta diversity describes the variation between two or more samples. Permutational multivariate analysis of variance (PERMANOVA) models were constructed to test the effect of each treatment on Beta diversity. Unweighted Bray-Curtis dissimilarity was computed to quantify the compositional differences between groups. The inter-relatedness of samples was visualised by principle components analysis (PCA), where samples with similar compositions cluster together closely.

6.3.5.4 Total abundance calculations

To allow a robust assessment of treatment effect on individual ASV abundance, total abundance was calculated by multiplying relative abundance of each ASV within a sample by the total 16S copies in that sample, as previously described.[153,342] An important methodological consideration is that some taxa have more than one copy of the 16S rRNA gene, with some species harbouring as many as 8 copies within their genome.[343] This clearly has potential to bias total abundance calculations. To account for this, the bioinformatics package PICRUSt2 (phylogenetic investigation of communities by reconstruction of unobserved states) was utilised to predict 16S rRNA gene count for each individual ASV. [344,345] ASV reads were then normalised to gene count before estimating relative abundance and multiplying by total sample 16S copy count.

6.4 Results

6.4.1 Sequence counts

Sequencing was performed for 63 samples (56 study samples, 3 negative kit controls, 2 positive kit controls, 1 negative sequencing control and 1 positive sequencing control) resulting in 1,115,862 reads. After filtering, denoising and merging, 909,633 sequences remained of which 16,302 were chimeric and were removed. The remaining 893,331 sequences were then taxonomically assigned to 847 individual ASVs.

Figure 6.4.1 illustrates the number of sequence reads for each study sample and also for the controls. The study sample with the most sequence reads had 23,352, whilst the sample with the least sequences reads had 273. There was a positive association ($r= 0.50$, $p<0.001$) between sequence reads and the sputum bacterial load as measured by qPCR (Log10 16S copies/ml), Figure 6.4.2.

Samples with less than 500 reads were excluded from further analyses meaning paired samples were available for 10 and 15 participants for the AZLI+IV and IV+IV treatment periods respectively. For between-treatment comparisons, 10 participants had a pair of samples for each treatment available for analysis (40 samples in total). 2/40 (5%) had <1000 reads and were therefore scrutinised for consideration of removal from further analysis. Visual inspection of these samples (sample ID: 6A2 and 4B2) confirmed similar community structures to those participants' other study samples and rarefaction curves showed they had approached asymptote sufficiently to be considered an accurate representation and were included for further analysis, see Figure 6.4.3.

Figure 6.4.2: Positive association between sequencing reads per sample and the bacterial load

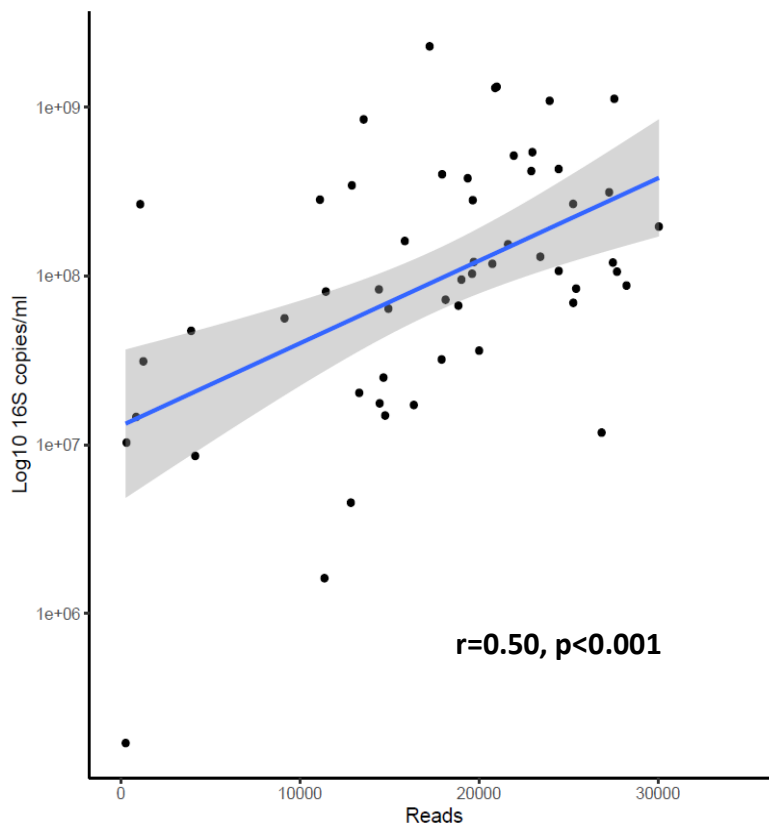


Figure 6.4.1: Distribution of sequencing reads per sample for all study samples and controls

NUNEG3=Sequencing negative control. NegControl1,2,3 =Negative kit controls.

PosControl&PosControl3=Positive kit controls. NUPOS3=Sequencing positive control

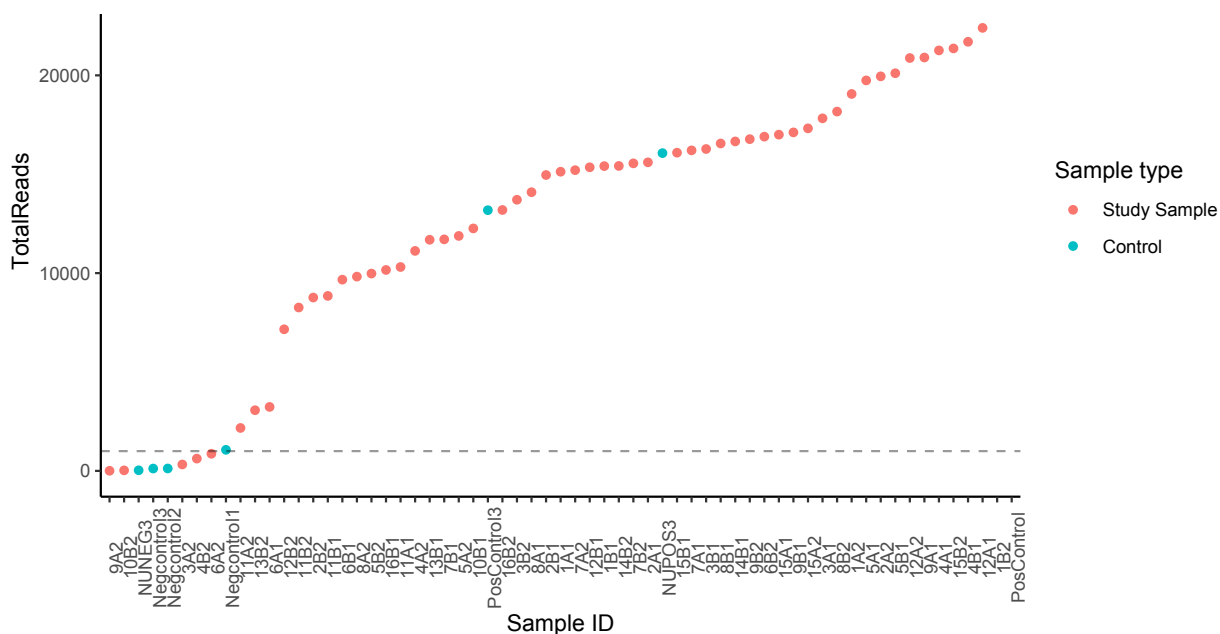
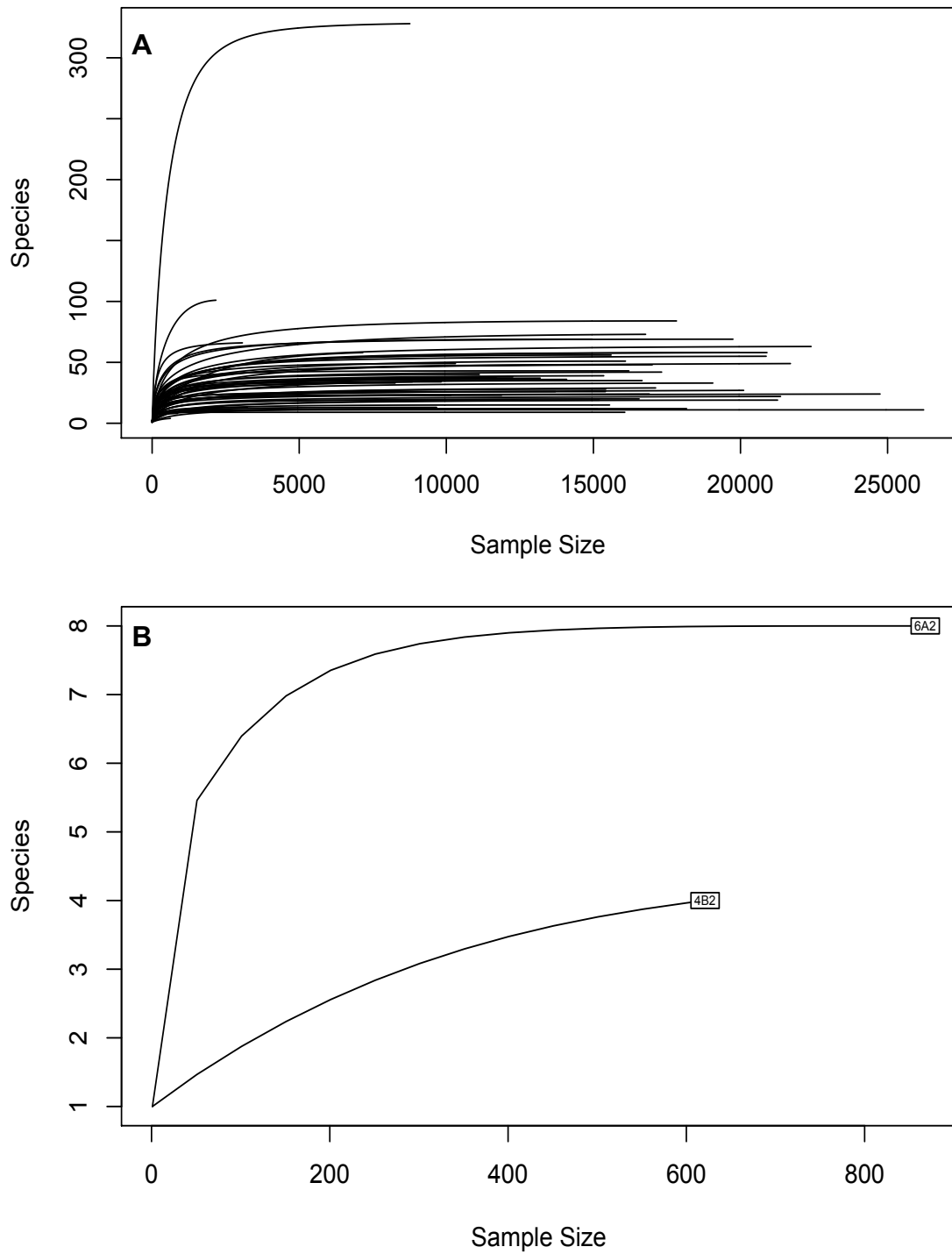


Figure 6.4.3: Rarefaction curve for all samples (A) showing asymptote was reached by ~1000 reads for the vast majority of samples. Two samples with <1000 reads were included for analysis after confirming asymptote was approached despite low reads (B).



6.4.2 Comparison of controls and clinical specimens

Significantly fewer reads were obtained in the negative controls than study specimens (median reads [IQR] 121 [97 to 358] vs. 15279 [9939 to 17166], $p < 0.001$). The sequencing negative control was dominated by *Escherichia coli*, see Figure 6.4.4, a pattern not seen in the clinical samples suggesting no major sequencing contamination of the clinical samples. The negative kit controls mainly consisted of ASVs resolved to the genera *Pseudomonas*, *Prevotella* and *Veillonella*, which in keeping with prior literature were the most common taxa in the clinical samples. [146,150,153,346] Given the negative kit controls had low overall reads and consisted mainly of taxa known to be prevalent in the CF lung microbiota with no evidence of known kit contaminants, it is likely that the reads represent carryover from clinical samples rather than significant kit contamination. [169] To assess for inherent bias within our extraction or sequencing methods, positive kit controls and a positive sequence control, all consisting of the same known ‘mock community’ were included. Figure 6.4.5 demonstrates all three positive controls clustered tightly and separately from negative controls. Figure 6.4.4 shows the positive controls all closely resembled each other, confirming consistency across both DNA extraction and sequencing. *Bacillus subtilis* only represents 12% of the mock community but represented ~25% of the sequenced microbiota, suggesting an extraction bias in favour of *Bacillus* spp. Reassuringly, *Bacillus* spp. were not found frequently in either negative controls or the core microbiota of study samples and hence any extraction bias is unlikely to have impacted results.

6.4.3 The CF lung microbiota is individualised with few clinical factors associated with community structure and diversity.

To test the hypothesis that clinical factors were associated with the CF lung microbiota, clinical characteristics and community structure/composition for each participant at study entry ($n=16$) were computed. Variables of interest were those considered to have biologic plausibility and/or those previously reported to be of significance in this setting. Table 6.4.1 shows differences in Richness and Shannon Diversity for each clinical characteristic with Bonferroni adjusted p -values.

Figure 6.4.4: Relative abundance of the top ten taxa in Positive Controls (Top Panel) and Negative Controls (Bottom Panel) used in the AZTEC-CF Study. All positive controls had >10,000 reads and all negative controls had <1,000 reads.

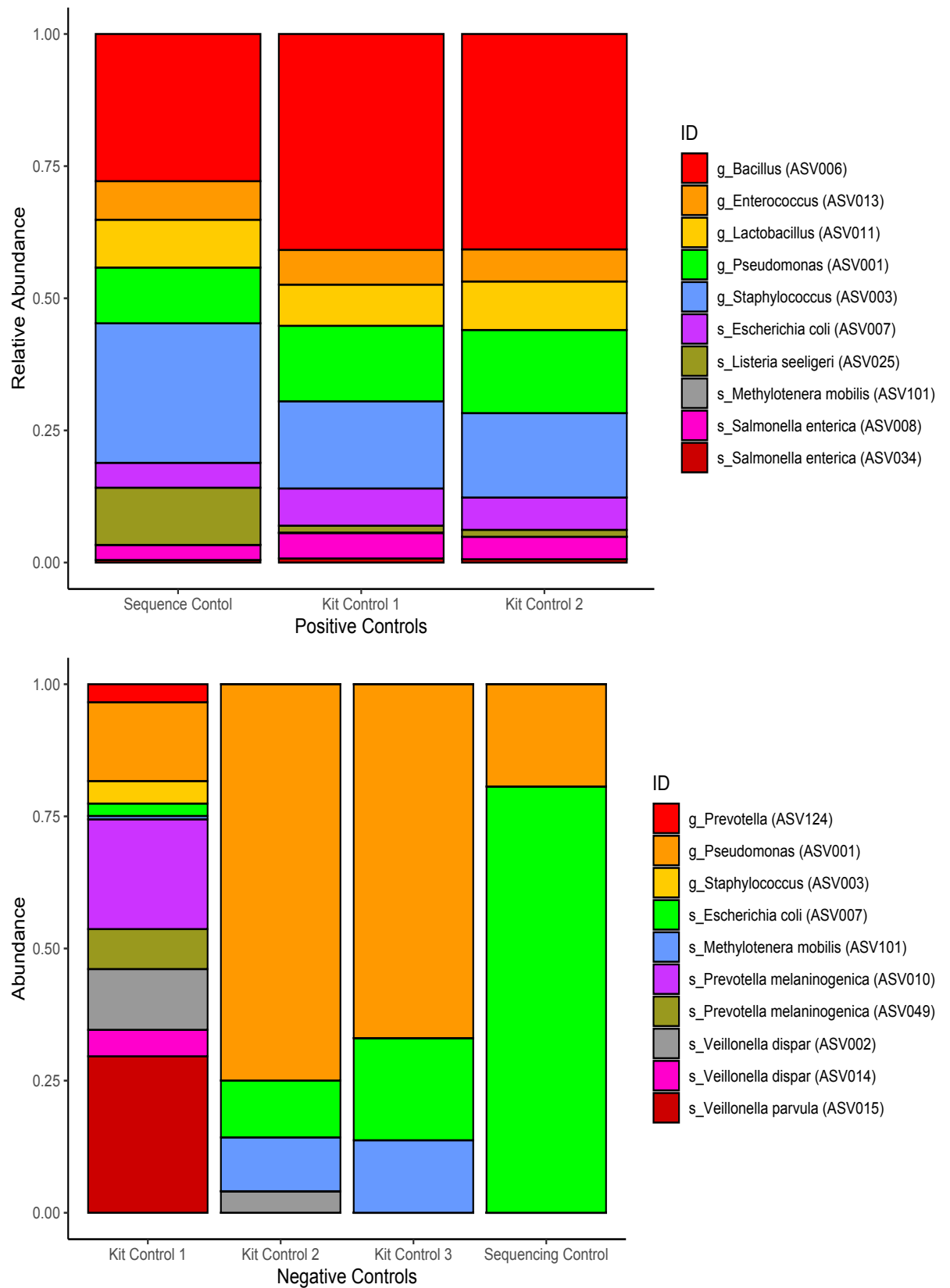
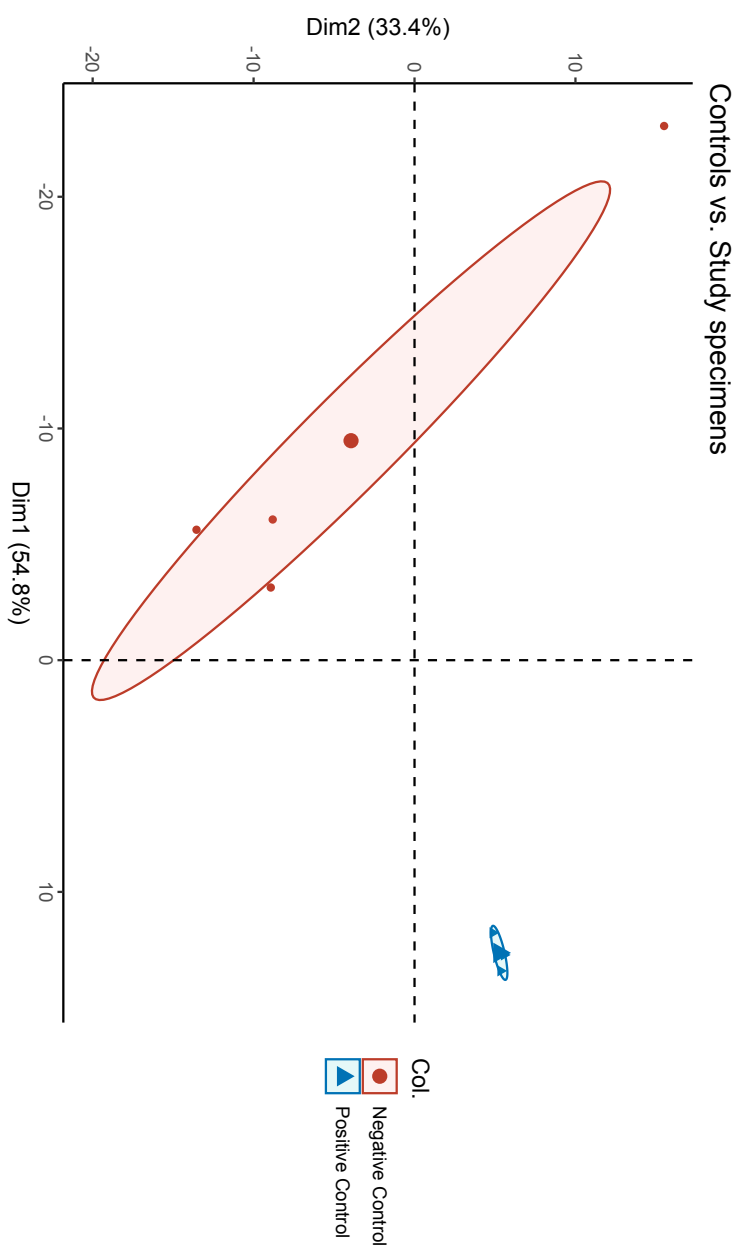


Figure 6.4.5: Principle components analysis of the inter-relatedness of negative and positive controls. Positive controls all closely resembled one another and clustered distinctly from negative controls which were markedly less similar to one another.



Overall, the CF sputum community structure was highly individualised with few consistent associations between community structure and clinical characteristics. No relationships between baseline clinical characteristics and richness were found, see Table 6.4.1 & Table 6.4.2. The presence of the Liverpool epidemic strain was associated with reduced Shannon diversity and the use of nebulised tobramycin was associated with increased Shannon diversity, see Table 6.4.2. No relationship was observed between age or lung function with richness or Shannon diversity, however this study is likely underpowered in that regard. When all study samples (n=56) were considered, both age ($r = -0.55$, $p < 0.001$) and bacterial load, i.e. total 16S copies ($r = -0.37$, $p = 0.004$) were inversely associated with Shannon diversity. A relationship was for allergic broncho-pulmonary aspergillosis (ABPA) whereby those participants with a previous diagnosis of ABPA (n=4) had greater Shannon diversity, indicated a more rich and even community.

6.4.4 At study entry the core microbiota was dominated by an ASV resolved to the *Pseudomonas* genus

The Prevalence Interval for Microbiome Evaluation (PIME) tool was used to identify a “core” microbiota. [341] The core microbiota consisted of 11 ASVs, which are presented in Table 6.4.3. The most abundant ASV was “ff9d93d7b7e46787568f2d241caeaf3b” which was resolved to the *Pseudomonas* genus (*g_Pseudomonas_ASV001*). Of the remaining 10 core constituents, 6 ASVs were resolved to the species level:

s_Escherichia_coil_ASV007,
s_Prevotella_melaninogenica_010
s_Rothia_dentocariosa_ASV039
s_Streptococcus_infantis_ASV018
s_Veillonella_dispar_ASV002
s_Veillonella_parvula_ASV017

Two were resolved to the *Streptococcus* genus (*g_Streptococcus*_ASV021 and *g_Streptococcus*_ASV023), one each to the *Granulicatella* genus and *Actinomyces* genus (*g_Granulicatella*_ASV033 and *g_Actinomyces*_ASV032 respectively).

The relative abundance of the core taxa for all participants at study-entry are presented in Figure 6.4.6. *g_Pseudomonas*_ASV001 was the most dominant taxa with >75% abundance in 11/16 (68.8%). *g_Pseudomonas*_ASV001 was more dominant in samples from participants known to harbour the Liverpool Epidemic Strain (median McNaughtey's Dominance index [95% CI]: 0.79 [0.62 to 0.92] vs. 0.69 [0.39 to 0.84], $p=0.0003$). For the 10 participants included in the comparative analyses, the core microbiota before and after each treatment are presented in Figure 6.4.7.

Table 6.4.1: Comparison of Shannon Diversity and Richness (i.e. number of species observed) across a number of clinical characteristics

Data are presented as median (interquartile range) with unadjusted and Bonferroni adjusted *p*-values. **= statistically significant after Bonferroni adjustment

Shannon Diversity				
	Condition Present	Condition Absent	p	Bonferroni
GORD	1.48 (0.569 to 2.13)	1.23 (1 to 1.67)	0.690	NS
CFRD	1.48 (0.59 to 1.76)	1.23 (0.569 to 1.9)	0.308	NS
ABPA	1.98 (1.19 to 2.45)	1.24 (0.59 to 1.67)	0.005	**
LES	1.36 (0.58 to 1.76)	1.68 (1.05 to 2.2)	0.001	**
MSSA	1.43 (0.904 to 2.04)	1.36 (0.59 to 1.9)	0.950	NS
PPI	1.57 (0.597 to 1.95)	1.24 (1 to 1.98)	0.633	NS
Tobi	1.69 (1.07 to 2.2)	1.34 (0.58 to 1.76)	0.001	**
Coli	1.46 (0.892 to 1.98)	1.19 (0.904 to 1.19)	0.954	NS

Richness				
	Condition Present	Condition Absent	p	Bonferroni
GORD	48 (13 to 58)	38 (23 to 48)	0.780	NS
CFRD	37 (36 to 37)	48 (13 to 49)	0.900	NS
ABPA	43 (36 to 53)	36 (13 to 59)	0.900	NS
LES	42.5 (12 to 53)	43 (35.5 to 58)	0.703	NS
MSSA	43 (36 to 48.5)	38 (12 to 66)	0.900	NS
PPI	43 (37 to 58.5)	42.5 (23 to 53)	0.503	NS
Tobi	42.5 (29 to 58)	43 (12 to 49)	0.870	NS
Coli	30 (12 to 30)	43 (36 to 53.5)	0.384	NS

Abbreviations: GORD=Gastro-oesophageal reflux disease; CFRD=Cystic fibrosis related diabetes; ABPA=Allergic bronchopulmonary aspergillosis; LES=Liverpool Epidemic Strain of *P. aeruginosa*; MSSA=Methicillin sensitive *S. aureus*; PPI=Proton pump inhibitor; Tobi=Nebulised Tobramycin; Coli=Nebulised Colistimethate

Table 6.4.2: Correlations of clinical characteristics with alpha diversity measures

Pearson's correlation coefficient with unadjusted and Bonferroni adjusted *p*-values

Shannon diversity			
	r	p	Bonferroni
Age	-0.45	0.080	NS
Log16S	-0.21	0.430	NS
BMI	0.03	0.920	NS
Annualised IV days	0.29	0.280	NS
Blood white blood count	0.42	0.110	NS
Blood C-reactive protein	0.21	0.440	NS
FEV1	-0.27	0.400	NS

Richness			
	r	p	Bonferroni
Age	-0.05	0.865	NS
Log16S	-0.05	0.845	NS
BMI	0.30	0.258	NS
Annualised IV days	-0.34	0.200	NS
Blood white blood count	0.03	0.909	NS
Blood C-reactive protein	-0.25	0.357	NS
FEV1	0.19	0.489	NS

Abbreviations: BMI=Body Mass index; IV=Intravenous antibiotic; FEV1=Forced expiratory volume in one second

Table 6.4.3: Taxonomic classification of the core bacterial constituents of the CF microbiota identified in the AZTEC-CF study.

ASV	Phylum	Class	Order	Family	Genus	Species	ASV_ID
ASV001	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		ff9c93d7b7e46787568f2d241caeef3b
ASV039	Actinobacteria	Actinobacteria	Actinomycetales	Micromonadaceae	Rothia	dentocariosa	d83f60183d81253a505beaee13cd168f
ASV010	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	melaninogenica	b7b4ecb07e1719860260746a5443092a
ASV021	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus		06f825b512d903b9230e1a55d87359ee
ASV023	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus		fd496fd32dc808ade2e8b6c9d8ee13d
ASV007	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherchia	coli	d46e2205f0c6ecf67b51f83d11c509c
ASV002	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veillonella	dispar	923f521b9cf13f1f95c9367e09bbc1c
ASV022	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Actinomyces		df8456a1abtbfb4c8a2c450b44378d4cb
ASV018	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	infantis	edc9e5c16e40aff1eadce6597940f08f
ASV017	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veillonella	parvula	cd9401a6bce4a63af516d06d2a843f9d
ASV033	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Granulicatella		2bd9309f2f97cae51d18d06ea1ca519a

Figure 6.4.6: The relative abundance of the core taxa at baseline for all AZTEC-CF study participants. Each bar represents the baseline sample for each participant with Study ID denoted above the respective bar. The relative abundance of all core taxa in each sample are represented by the proportion their respective colour occupies within the bar.

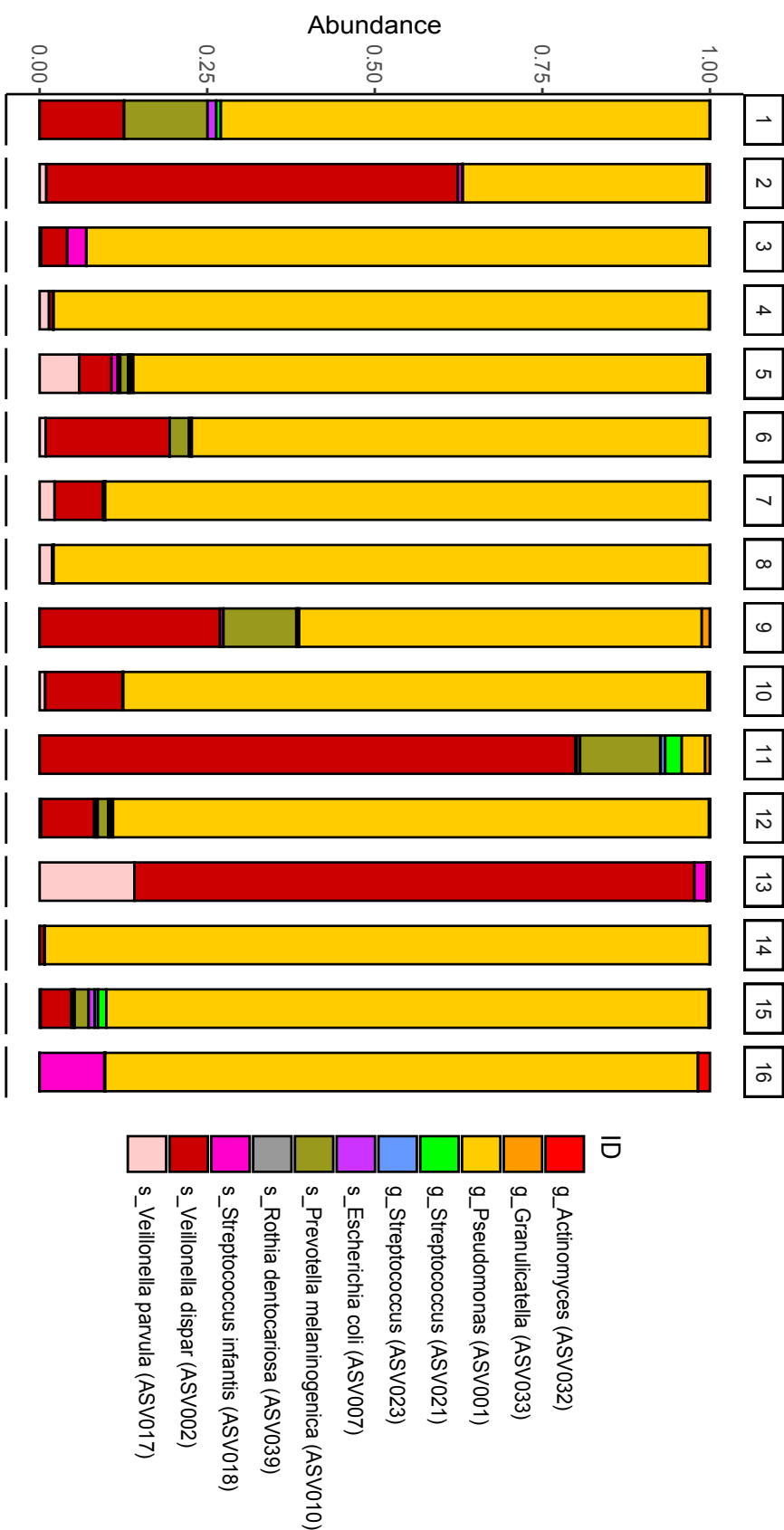
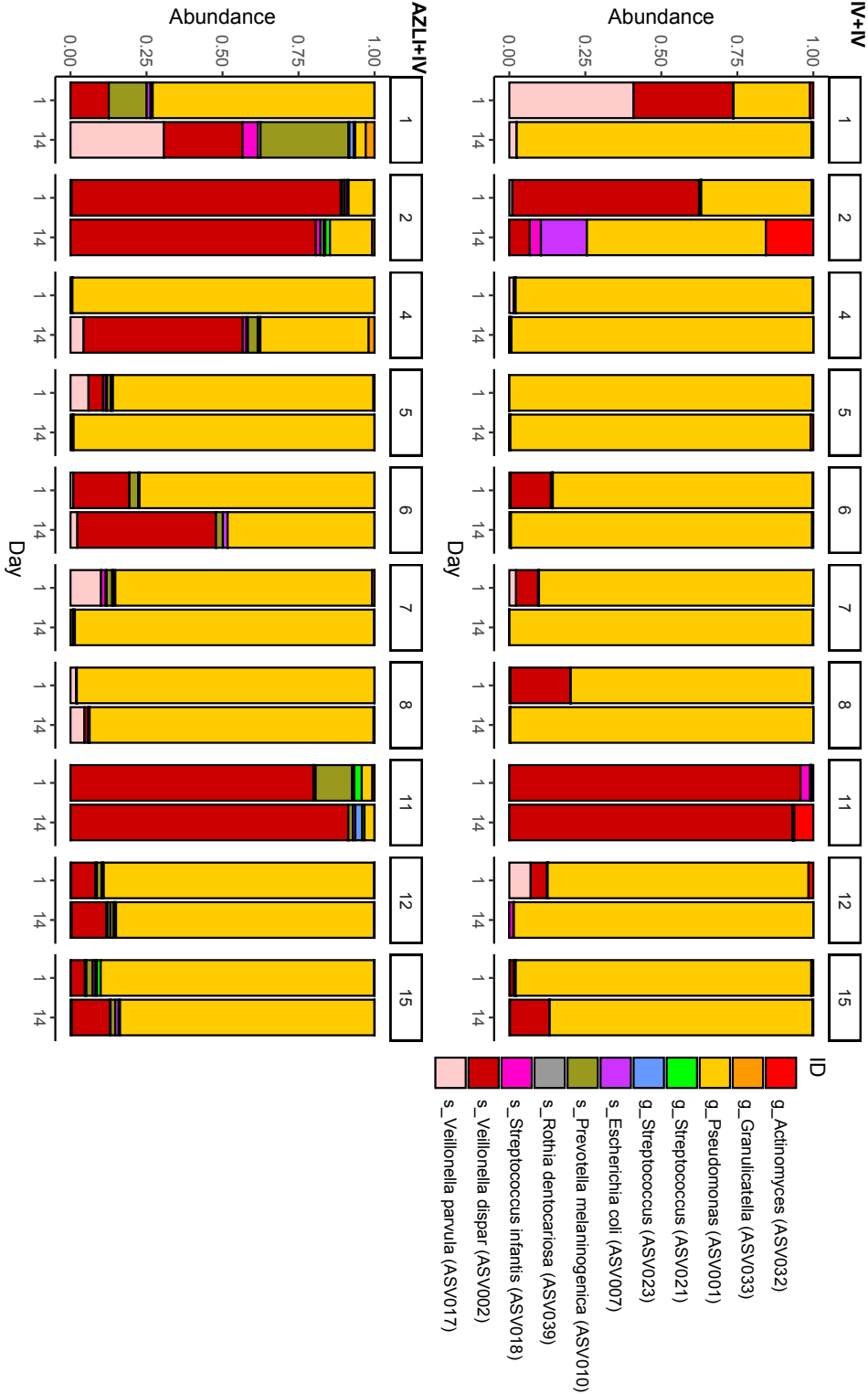


Figure 6.4.7: Relative abundance of core taxa before and after each treatment. Each bar represents a sputum sample for each participant with Study ID denoted above the respective bar. Samples from exacerbations treated with IV+IV are in the top row and AZLI+IV in the bottom row. On the x-axis Day 1 or Day 14 is denoted. The relative abundance of all core taxa in each sample are represented by the proportion their respective colour occupies within the bar.



6.4.5 The CF lung microbiota is highly resilient to exacerbation and treatment

Richness, Shannon Index and Fisher's alpha index were calculated for all 53 included samples. Overall, all measures of alpha diversity were preserved across the study period suggesting the CF microbiota community structure is resilient to perturbation at the time of exacerbation and also with treatment, see Figure 6.4.8. Similarly, when each treatment was considered separately, neither IV+IV, nor AZLI+IV were associated with any significant changes in alpha diversity measures, see Figure 6.4.8

6.4.6 IV+IV but not AZLI+IV exert significant changes on CF sputum community composition

At day 1 i.e. pre-treatment, there was no significant difference between the two treatment groups, (Bray-Curtis $r^2=0.08$, $p=0.21$, see Figure 6.4.9). After 14 days treatment, there were significant differences between treatments (Bray-Curtis $r^2=0.18$, $p=0.001$, see Figure 6.4.9). These findings were supported by comparing beta diversity at day 1 and day 14 for each treatment, where no change was seen for the AZLI+IV arm (Bray-Curtis $r^2=0.03$, $p=0.64$) but was observed in IV+IV treatment group (Bray-Curtis $r^2=0.14$, $p=0.02$).

6.4.7 AZLI+IV and IV+IV exert different effects on individual ASVs

To investigate the effect of each treatment at the individual ASV level, the total abundance of individual constituents of the core microbiota were calculated on day 1 and day 14 for each treatment. The IV+IV treatment was associated with significant reductions in total abundance of *s_Veillonella_parvula*_ASV017 (Day 1 median [IQR] abundance 6.8 [6.3 to 7.3] vs Day 14 0.0 [0.0 to 6.1] Log₁₀ copies/ml, paired Wilcoxon $p=0.01$), *s_Veillonella_dispar*_ASV002 (5.8 [5.2 to 6.4] vs. 0.0 [0.0 to 3.2] Log₁₀ copies/ml, paired Wilcoxon $p=0.009$), *g_Granulicatella*_ASV033 (4.8 [2.0 to 7.7] vs. 0.0 [0.0 to 5.5] Log₁₀copies/ml, paired Wilcoxon $p=0.04$) and *s_Prevotella_melaninogenica*_ASV010 (6.2 [3.9 to 8.9] vs. 0.0 [0.0 to 0.0], paired Wilcoxon $p=0.02$), see Figure 6.4.10.

AZLI+IV was associated with an increase in the total abundance *g_Streptococcus_ASV023* (median abundance [IQR] 4.4 [1.9 to 7.0] to 5.1 [4.9 to 5.4] Log₁₀ copies/ml, paired-Wilcoxon $p=0.018$), but no other significant differences. Neither IV+IV, nor AZLI+IV (7.5 [6.1 to 8.3] vs. 7.3 [6.1 to 7.9] Log₁₀ copies/ml, paired Wilcoxon $p=0.18$) was associated with any overall difference in *g_Pseudomonas_ASV001*.

Between-treatment differences at day 14 were most pronounced for *s_Prevotella_melaninogenica_ASV010* and *s_Veillonella_dispar_ASV002*. Median [IQR] change in total abundance of *s_Prevotella_melaninogenica_ASV010* for AZLI+IV was +0.1 [-3.5 to 1.3] Log₁₀ copies/ml vs. -5.7 [-6.7 to 0.0] Log₁₀ copies/ml for IV+IV, paired Wilcoxon $p=0.049$. For *s_Veillonella_dispar_ASV002*, change in total abundance for AZLI+IV was +0.28 [-0.15 to +0.73] Log₁₀ copies/ml vs. -6.1 [-7.5 to 0.0] Log₁₀ copies/ml for IV+IV, paired Wilcoxon $p=0.049$, see Figure 6.4.11.

Figure 6.4.8 Top panel: Alpha diversity measures (Richness, Shannon Index and Fisher's Alpha Diversity index) across the AZTEC-CF Study period. . Bottom two panels: Alpha diversity measures before and after AZLI+IV (orange) and IV+IV (blue) .

Timepoint 1=Exacerbation 1 Day 1, Timepoint 2: Exacerbation 1 Day 14, Timepoint 3=Exacerbation 2 Day 1, Timepoint 4 = Exacerbation 2 Day 14)

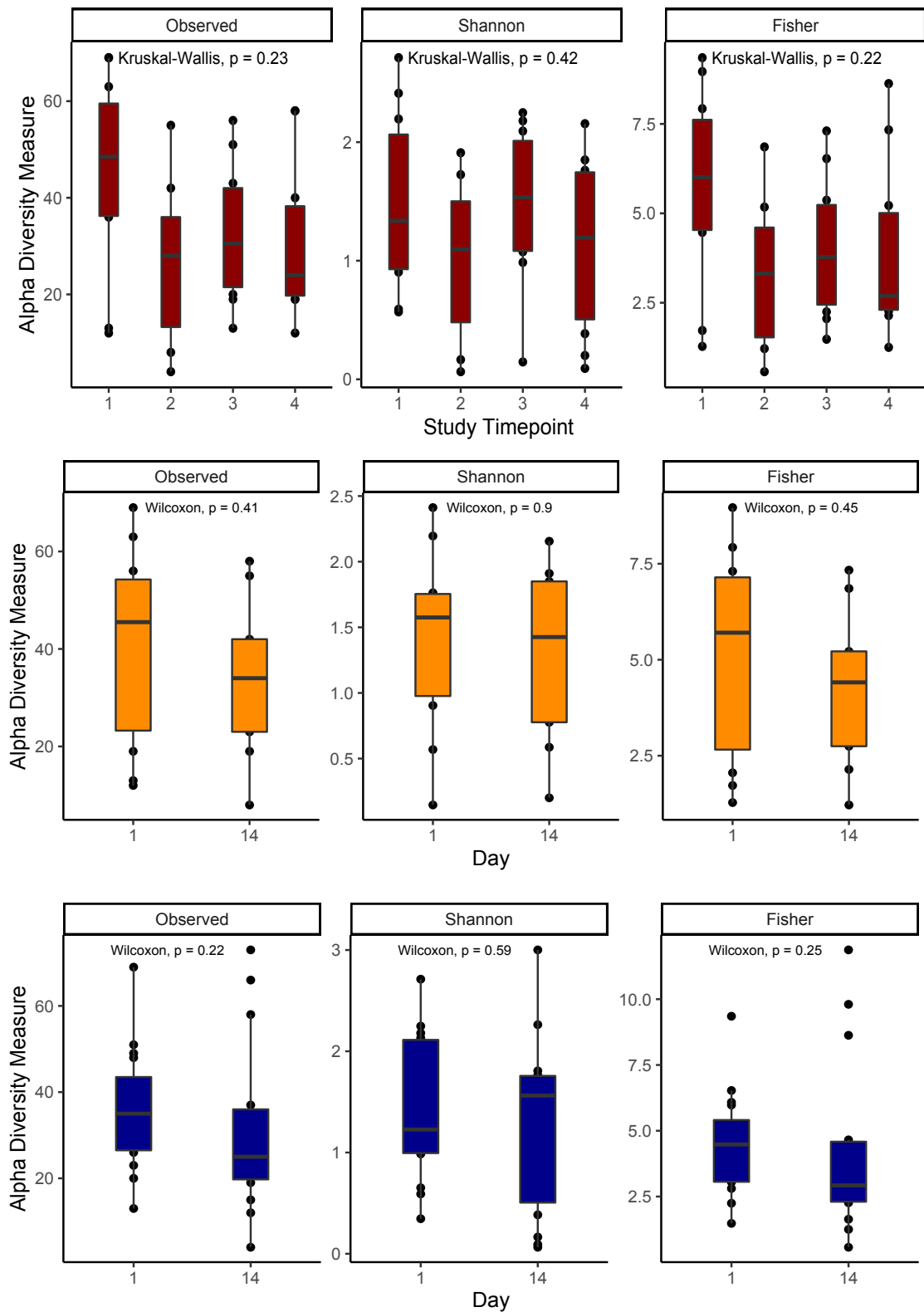


Figure 6.4.9: Principle component analysis comparing inter-relatedness of samples before and after each treatment.

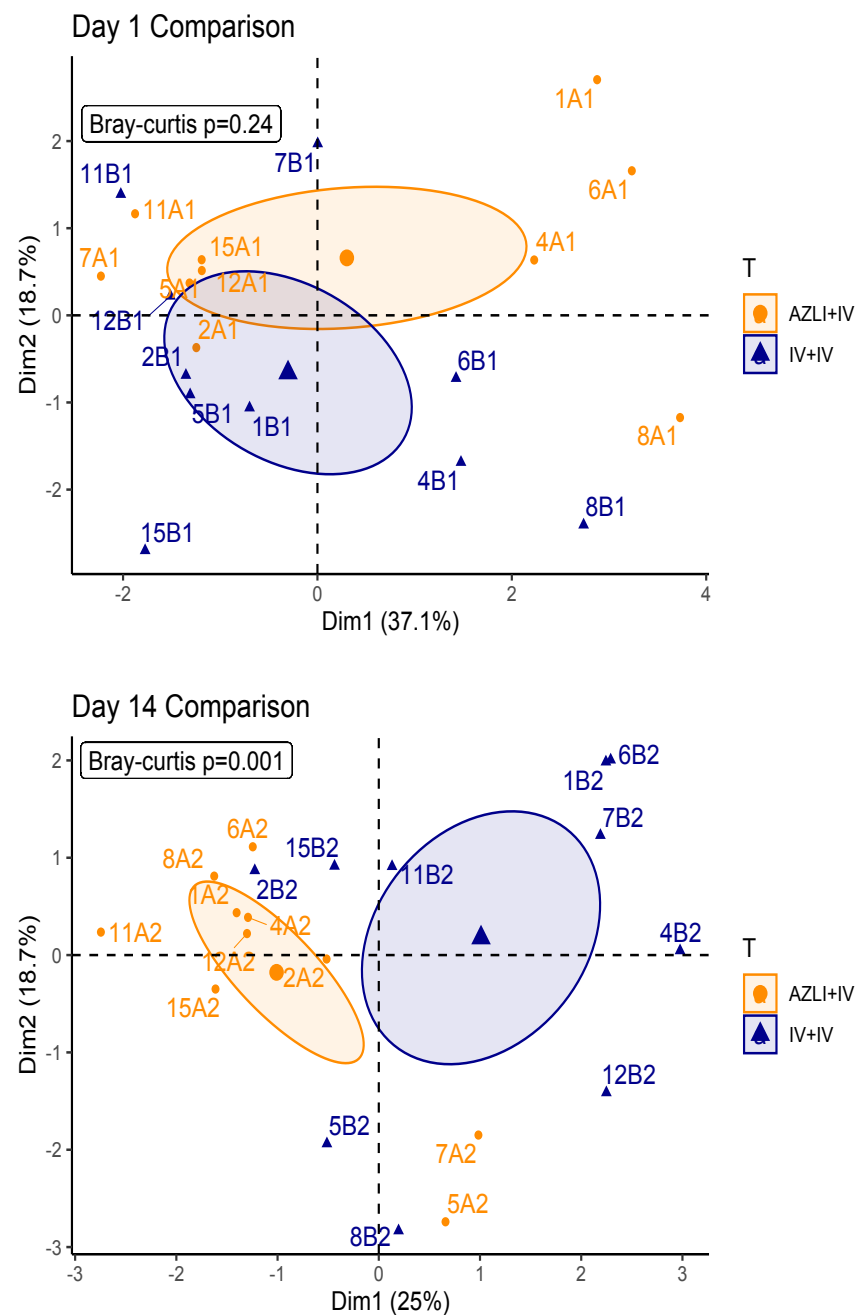


Figure 6.4.10: Boxplots of total abundance of each core ASV on Day and Day14 for IV+IV (Dark blue) and AZLI+IV (Orange).

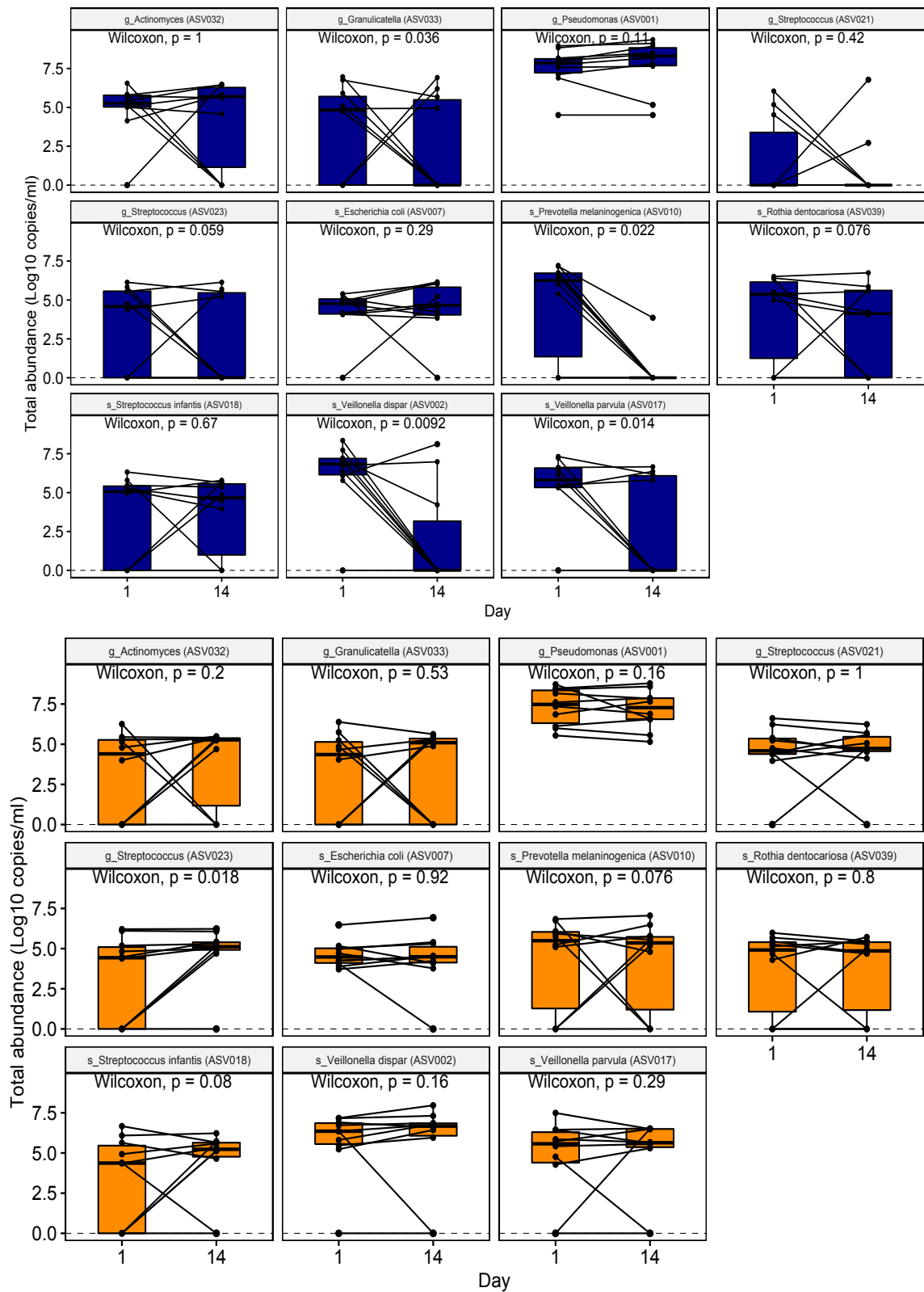
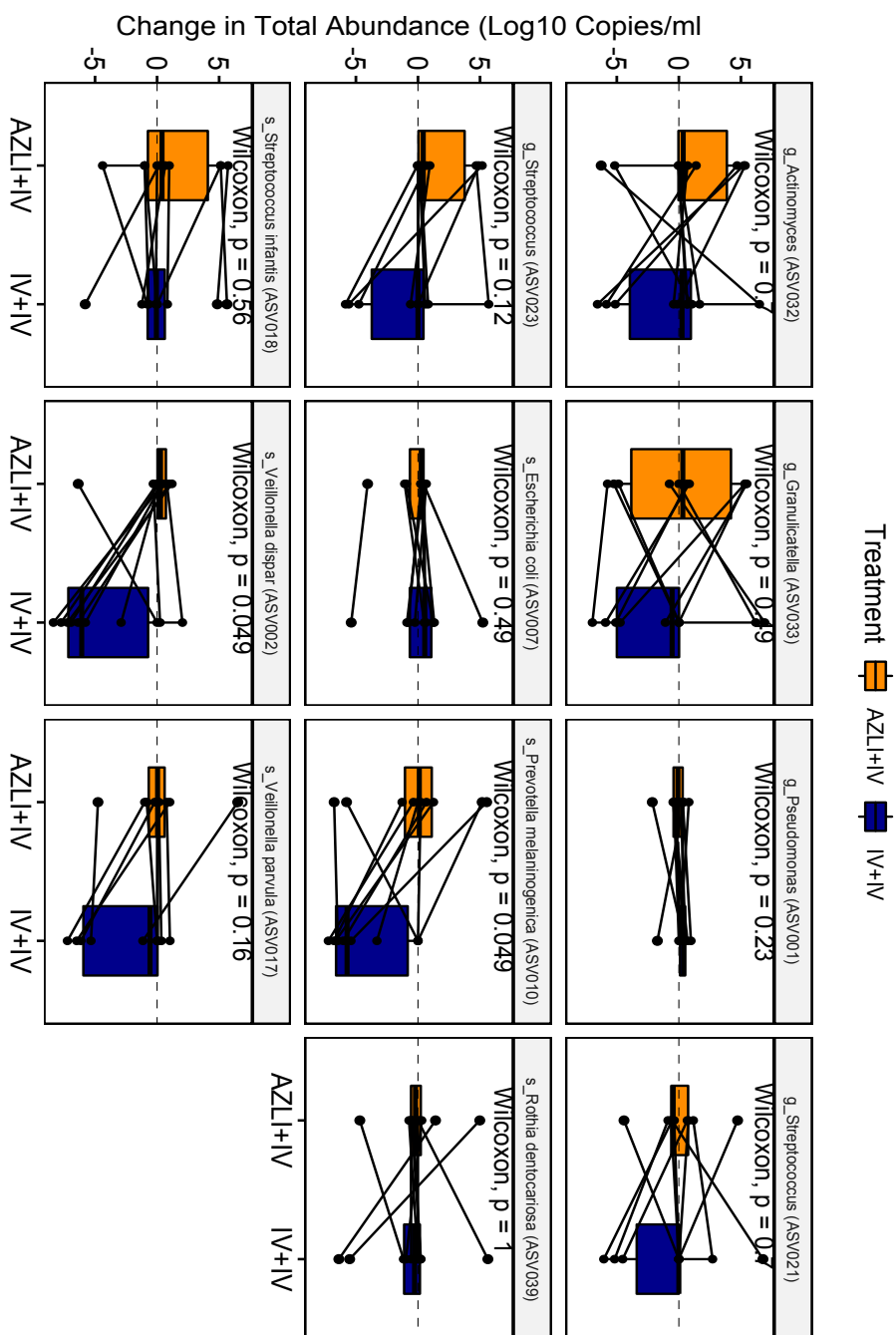


Figure 6.4.11: Boxplots of between-treatment differences for change in total abundance of each core ASV at day 14



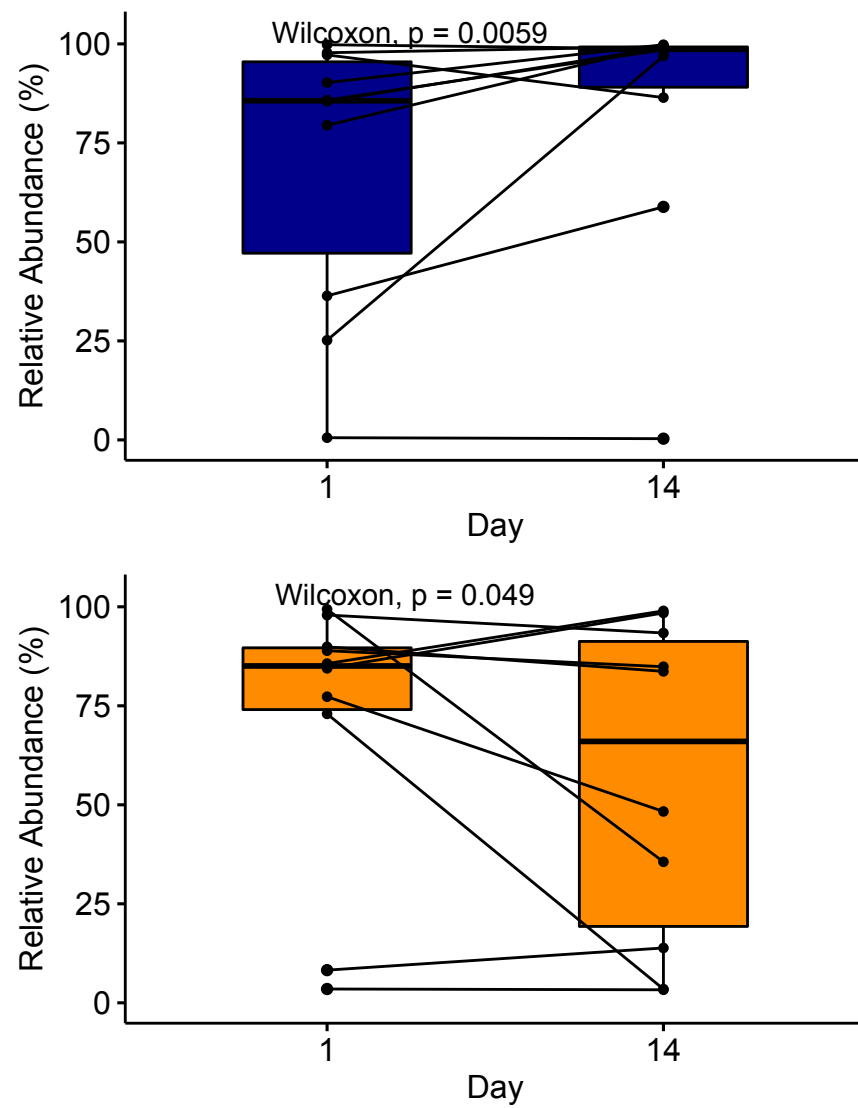
6.4.8 Changes in individual ASVs analysed by relative abundance alone can be misleading

To investigate whether the analysis of total abundance rather than relative abundance of individual ASVs provides extra insight or leads to substantially different conclusions, the analyses performed in section 6.4.7 were repeated using relative abundance data rather than total abundance.

When analysis was performed using relative abundance only, the abundance of *g_Pseudomonas_ASV001* was seen to increase with IV+IV (median [IQR] day 1 vs. day 14: 85.6% [61.5 to 100] vs. 98.6% [93.5 to 100], $p=0.006$), whereas decreases were observed for AZLI+IV (median [IQR] day 1 vs. day 14: 85.1% [77.3 to 92.8] vs. 66.0% [30.0 to 100], $p=0.05$), see Figure 6.4.12. In each case, the total abundance of *g_Pseudomonas_ASV001* was static, see section 6.4.7, and the relative changes were therefore actually as a result of absolute changes in other ASVs, for example reduced *s_Prevotella_melaninogenica_ASV010* and *s_Veillonella_dispar_ASV002* with IV+IV treatment, and increased *g_Streptococcus_ASV023* with AZLI+IV. These findings serve to highlight the limitations in drawing conclusions from relative abundance data alone.

Figure 6.4.12: Boxplots of relative abundance of *g_Pseudomonas_ASV001* at day 1 and day 14 for IV+IV (Dark blue) and AZLI+IV (Orange).

Comparison by Wilcoxon Signed-Rank test



6.4.9 Changes in *Pseudomonas* abundance within the lung microbiota are associated with quality of life outcomes

To explore whether changes in particular elements of the lung microbiota were associated with different clinical outcomes, the relationship between the lung microbiota and changes in lung function, quality of life and time to next exacerbation was compared.

First, the relationship between changes in individual ASVs and clinical outcomes was explored. A moderate positive correlation was observed for change in *g_Granulicatella_ASV033* abundance and change in lung function, $r=0.53$, $p=0.02$, however this was not significant when Bonferroni correction was applied and no other relationship between ASVs and changes in lung function were observed, see Figure 6.4.13.

Associations between changes in ASV abundance and quality of life (CFQ-R Respiratory domain) are presented in Figure 6.4.14. A moderate negative correlation was observed between change in total *g_Pseudomonas_ASV001* abundance and changes in quality of life ($r=-0.61$, $p=0.004$, remains significant with Bonferroni correction) but no associations were found for other taxa. This pattern was similar for both IV+IV and AZLI+IV treatments, see Figure 6.4.14B and also when changes were considered in terms of relative abundance as well as total abundance, Figure 6.4.14C.

To explore if changes in the abundance of ASVs were related to time to next exacerbation, a Cox proportional hazard model was constructed for each ASV and the results are presented in Table 6.4.4. Changes in ASV abundance over the course of each treatment were not found to be associated with time to next exacerbation.

Figure 6.4.13: Correlation between changes in total abundance of core ASVs and change in lung function at day 14

Raw p -values are presented. A Bonferroni adjusted value of $p < 0.0045$ was considered statistically significant

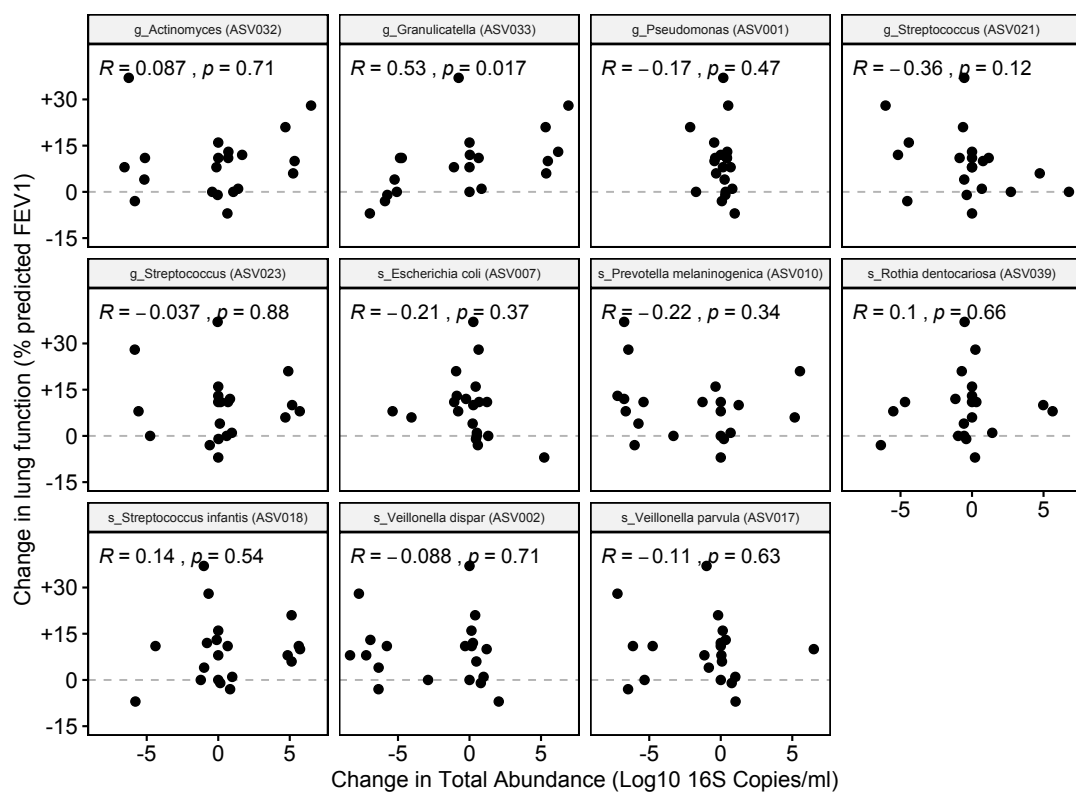


Figure 6.4.14: Correlation between changes in total abundance of core ASVs and CFQ-R respiratory domain (A). A negative correlation was seen for *g_Pseudomonas_ASV001*, which was consistent for both total abundance (B) and relative abundance (C).

Raw *p*-values are presented. A Bonferroni adjusted value of $p < 0.0045$ was considered statistically significant for panel A.

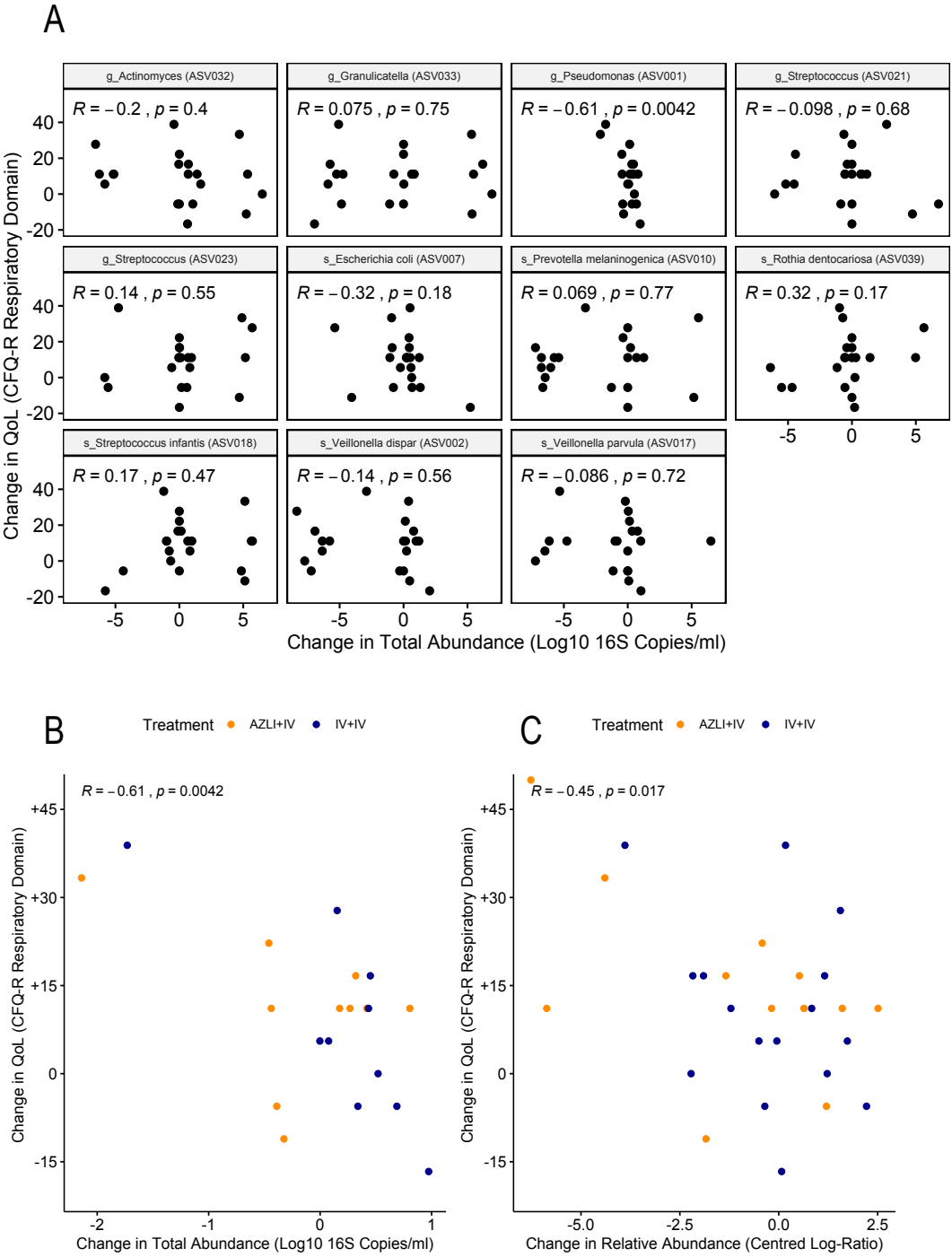


Table 6.4.4: Cox proportional hazards models constructed for the relationship between change in individual ASV abundance from day 1 to day 14, and time to next exacerbations.

ID	Hazard Ratio (95% Confidence Interval)	<i>p</i>	<i>Bonferroni corrected</i>
<i>g_Pseudomonas</i> _ASV001	1.2 (0.65 to 2.21)	0.56	NS
<i>s_Streptococcus_infantis</i> _ASV018	0.88 (0.77 to 1)	0.05	NS
<i>g_Granulicatella</i> _ASV033	0.99 (0.89 to 1.09)	0.82	NS
<i>s_Veillonella_dispar</i> _ASV002	0.94 (0.83 to 1.06)	0.31	NS
<i>s_Prevotella_melaninogenica</i> _ASV010	0.9 (0.8 to 1.01)	0.07	NS
<i>s_Veillonella_parvula</i> _ASV017	0.94 (0.82 to 1.08)	0.38	NS
<i>s_Escherichia_coli</i> _ASV007	1.08 (0.89 to 1.31)	0.42	NS
<i>s_Rothia_dentocariosa</i> _ASV039	0.97 (0.83 to 1.14)	0.69	NS
<i>g_Actinomyces</i> _ASV032	0.96 (0.86 to 1.07)	0.49	NS
<i>g_Streptococcus</i> _ASV023	0.87 (0.75 to 1.01)	0.07	NS
<i>g_Streptococcus</i> _ASV021	0.86 (0.7 to 1.07)	0.17	NS

Next, the relationships between bacterial loads/abundances on day 1 of treatment, and treatment outcomes were examined. Overall, no relationship was seen between day 1 total bacterial load and change in lung function ($r= 0.23$, $p=0.33$) or change in the CFQ-R Respiratory domain ($r= -0.08$, $p=0.72$). Similarly, no relationships were observed when each treatment was considered separately.

The associations between the abundance of individual core ASVs on day 1 and subsequent changes in lung function are presented in Figure 6.4.16. *s_Prevotella_melaninogenica* was the only ASV with a statistically significant association ($r= -0.52$, $p=0.018$), however the association was no longer significant when bonferroni adjustment was applied. When each treatment was considered separately, baseline *s_Prevotella_melaninogenica* abundance appeared to be more important to outcomes of IV+IV ($r= -0.7$, $p=0.025$) than AZLI+IV ($r= -0.33$, $p=0.36$), see Figure 6.4.17. To determine whether *s_Prevotella_melaninogenica* abundance is important for time to next exacerbation, day 14 samples were reviewed and assigned into a “High” or “Low” abundance group. The “Low” abundance group consisted of all those day 14 samples with $<1 \text{ Log}_{10} 16S \text{ copies/ml}$ (12/20 in total). The “High” abundance group was the remaining 8 samples. The median time to next exacerbation was 96 days in the “Low” abundance group, compared to 188 days in the “High” abundance group. Despite the clinically significant numerical difference, this was not statistically significant ($p=0.12$), see Figure 6.4.17.

Finally, the relationships between baseline abundance of ASVs and subsequent changes in quality of life are presented in Figure 6.4.18. No significant relationships were observed for any ASV once corrected for Bonferroni.

Figure 6.4.15: Correlation between qPCR derived total bacterial load on day 1 and subsequent clinical outcomes for each treatment

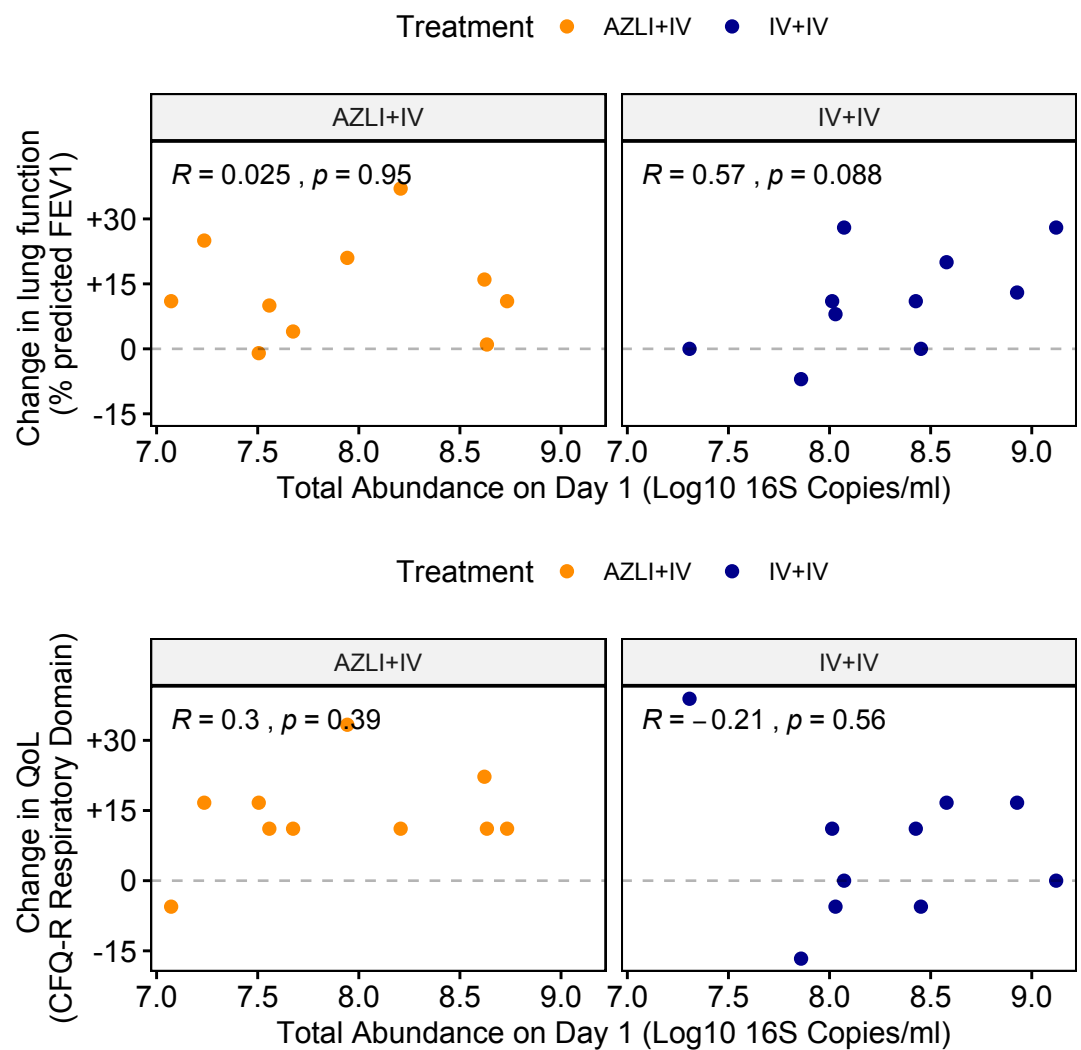


Figure 6.4.16: Correlation between total abundance of individual ASVs on Day 1 and subsequent changes in lung function

Raw p -values are presented. A Bonferroni adjusted value of $p < 0.0045$ was considered statistically significant

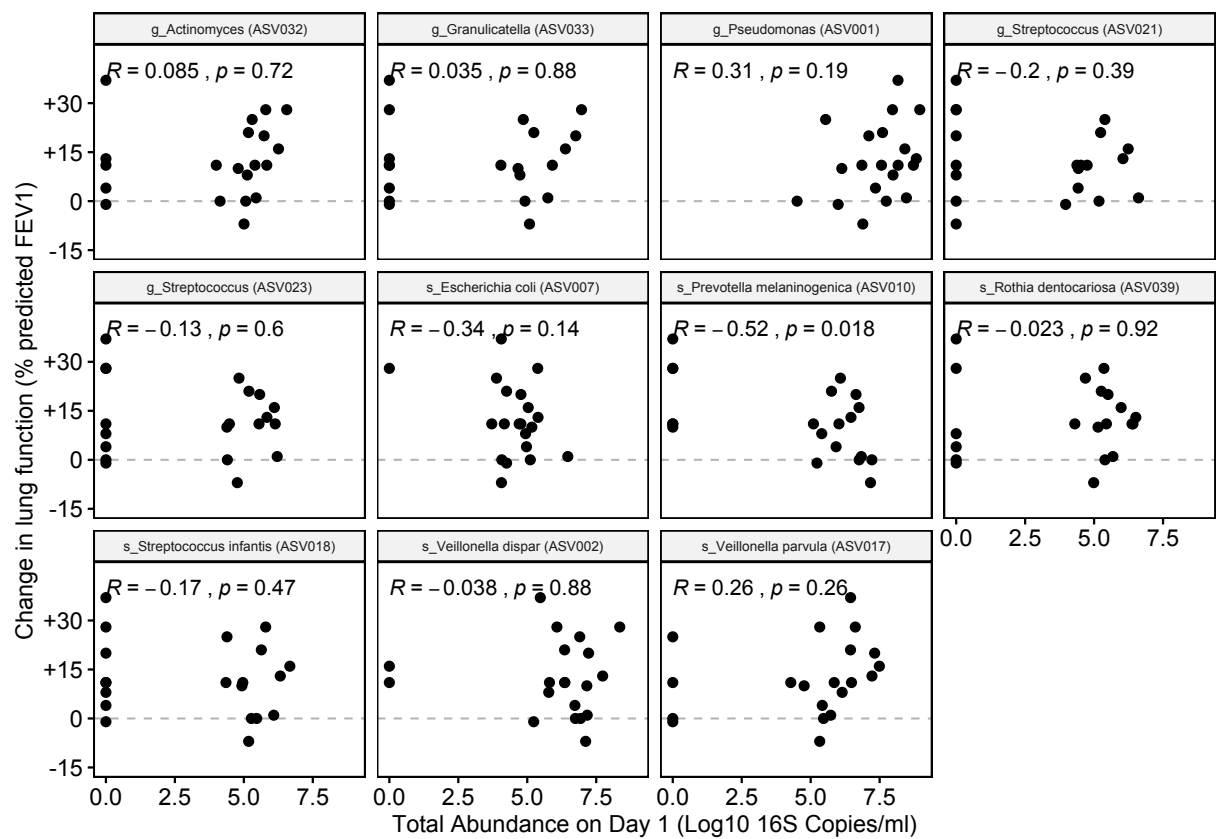


Figure 6.4.17: Correlation between the abundance of *Prevotella melaninogenica* on Day 1 and subsequent changes in lung function after treatment with 14 days of AZLI+IV and IV+IV. Below is a Kaplan-Meier plot for time to next exacerbation between those with high and low *Prevotella melaninogenica* abundance.

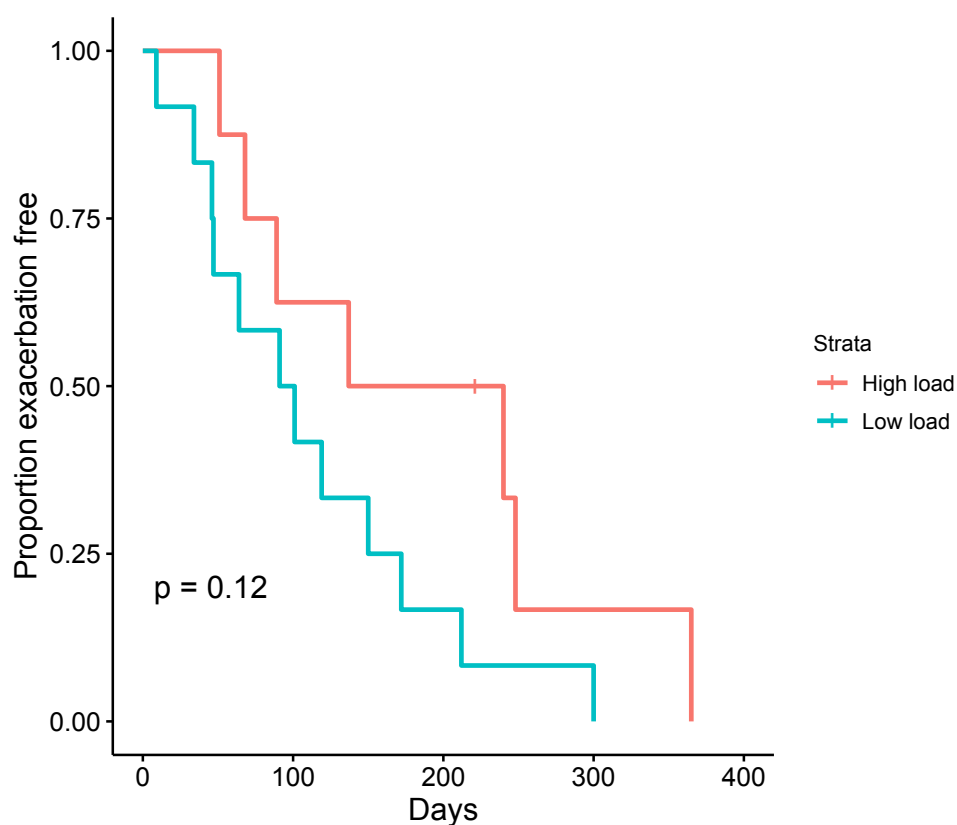
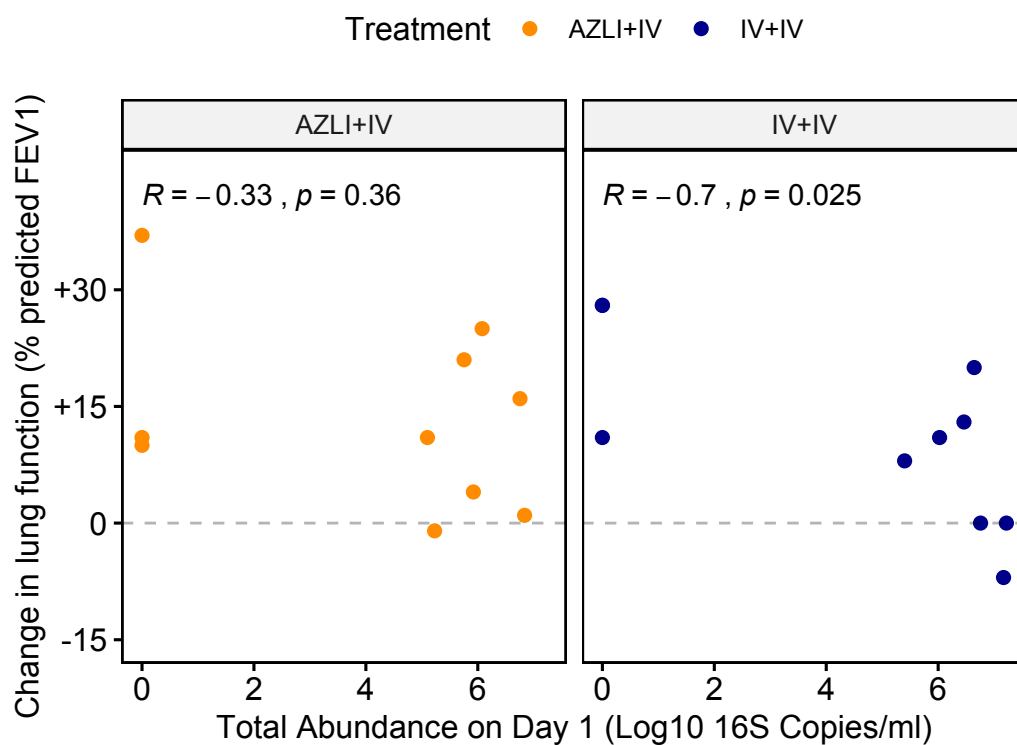
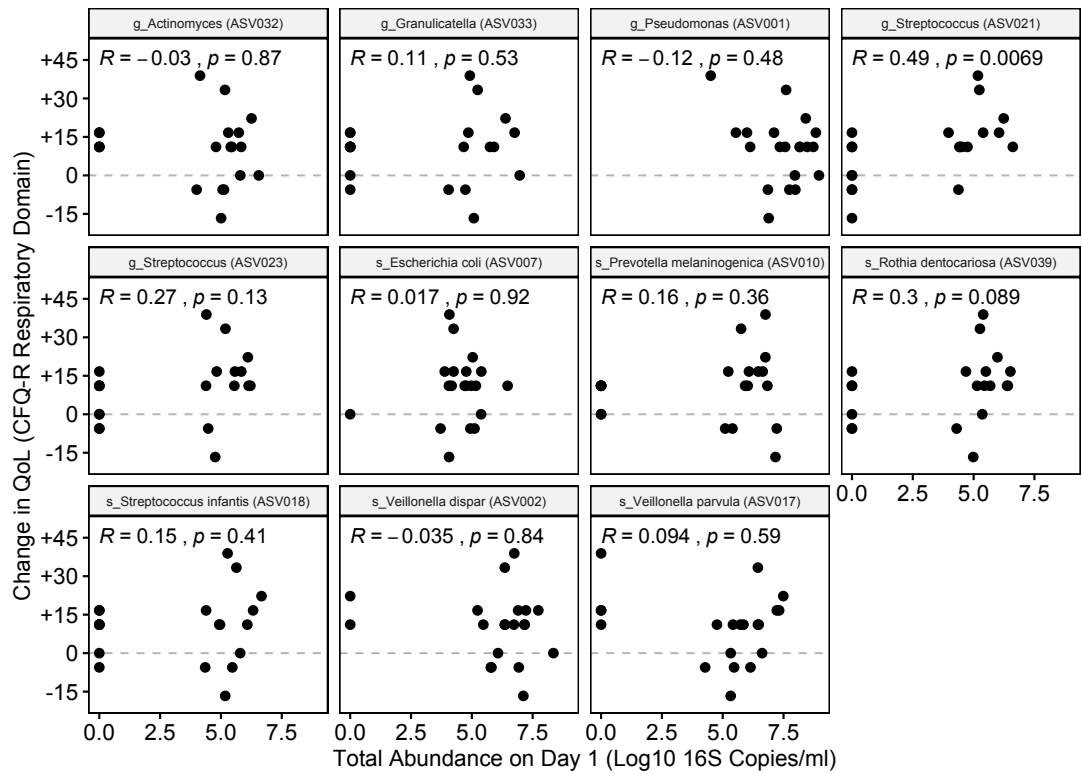


Figure 6.4.18: Correlation between abundance of individual ASVs on day 1 and subsequent change in quality of life

Raw p -values are presented. A Bonferroni adjusted value of $p < 0.0045$ was considered statistically significant



6.5 Discussion

This aim of this study was to explore the lung microbiota of AZTEC-CF study participants and investigate differences in the effect of AZLI+IV and IV+IV treatment approaches. Unsurprisingly, given the AZTEC-CF study was limited to adults with CF and known *P. aeruginosa* infection, the microbiota of study participants tended to be dominated by an ASV resolved to the *Pseudomonas* genus, and this was particularly the case in those known to be infected with the Liverpool Epidemic Strain (LES). A more diverse microbiota was observed in those without LES and also in those with a prior diagnosis of ABPA.

In keeping with prior literature, the core microbiota was enriched with taxa belonging to the *Prevotella*, *Veillonella* and *Streptococcus* genera. [127,146,150,153,347] These taxa are not considered classical CF pathogens, yet all are well documented as common constituents of the CF microbiota and associated with variable clinical outcomes. [151,340,348–350] In contrast to some prior studies, *Staphylococcus* was not found to be a constituent of the core lung microbiota in this study. This was surprising given *S. aureus* is a well-recognised CF pathogen and *Staphylococcus* OTUs have previously been reported in North American studies to be important for outcomes to inhaled aztreonam and inhaled tobramycin in the stable disease setting. [146,351] However, in keeping with the findings in this study, other studies in the UK utilising similar core partitioning techniques did not find *Staphylococcus* to be constituents of the core microbiota. [150,340,352] A potential explanation for this lies in the historical usage of anti-staphylococcal antibiotics in the UK as compared to North America, although demographic differences between study cohorts cannot be ruled out.

When IV+IV and AZLI+IV were compared, the two treatments exerted different effects on the lung microbiota. For example, although alpha diversity was maintained throughout the study, the beta-diversity of sputum samples differed after each treatment, despite samples coming from the same patients. The most striking

differences were reductions in the abundance of the anaerobes *Prevotella melaninogenica* and *Veillonella dispar* seen with IV+IV but not AZLI+IV. Interestingly, neither treatment had any discernible consistent effect on *Pseudomonas* abundance, despite this classically being the target of treatment in CF exacerbations.

This is the first study of the impact of an inhaled antibiotic on the CF lung microbiota in a controlled clinical trial setting. Of the currently available inhaled antibiotics, AZLI has received the most attention in terms of prior microbiome studies with previous work including a retrospective analysis using biobanked samples, [147] and also a prospective observational study. [146] Our study confirms the findings of those earlier studies in that AZLI was not consistently associated with perturbations of alpha diversity or changes in *Pseudomonas* abundance. The results here did differ in that changes in *Pseudomonas* abundance were associated with improved quality of life at day 14, a pattern not seen previously. Instead, the earlier studies suggested species belonging to the *Prevotella* and *Streptococcus* genera were more important when it came to changes in quality of life. The differences here may be related to the different study setting (i.e. acute exacerbation vs. clinically stable) or methodological differences discussed later.

The relative lack of impact of AZLI+IV on anaerobes may potentially be explained by the limited anaerobic cover provided by aztreonam and colistin (the two antibiotics in the AZLI+IV treatment), whereas the second IV antibiotic in the IV+IV arm was commonly a beta-lactam such as meropenem or piperacillin/tazobactam, which do convey greater anaerobic coverage. [353] However, AZLI use in clinically stable patients has previously been reported to result in reduced *Prevotella* abundance, suggesting some in-vivo activity may exist. [147] An alternative explanation is that nebulised antibiotics can only penetrate areas of the lung where there is good ventilation. If anaerobes were found more frequently in areas with poorer ventilation, AZLI would have less chance to act against them. Increased mucus plugging and subsequent reduced ventilation to some areas of the lung during an exacerbation could therefore explain the relative lack of impact against anaerobes

compared to IV+IV, which would reach poorly ventilated areas via the pulmonary vasculature.

The clinical relevance of the between-treatment differences as regards these anaerobic species is not entirely clear. For example, *Prevotella* identified in prior CF microbiome studies were associated with milder lung disease and reduced inflammation and was put forward as a marker of a more diverse and “healthy” microbiota. [127] Reductions could, on that basis, be considered undesirable yet elsewhere increases in *Prevotella* abundances have been reported at the time of exacerbation and subsequent reductions in *Prevotella* with treatment were associated with lung function improvement. [354–356] Part of the difficulty in making comparisons between studies relates to the poor ability of conventional 16S rRNA gene sequencing to taxonomically resolve beyond the genus level. Thus, the conflicting findings reported previously may be due to the sequences in question representing different *Prevotella* species within a heterogenous genus. [357]

More recently, both *Prevotella melaninogenica* and *Veillonella dispar* were noted to have increased prevalence in milder CF lung disease. [352] The authors speculated that interventions promoting the growth of those taxa might mitigate the effects of resident pathogens on lung function. The lack of effect on AZLI+IV on these species could therefore be considered advantageous. However, results here showed a trend towards greatest improvements in lung function in those with lower abundance of *Prevotella melaninogenica* at treatment initiation. Interestingly, after treatment, those with low *Prevotella melaninogenica* abundance showed a trend toward reduced time to exacerbation, although the small numbers in this study limit the interpretation of this observation. These results may suggest that community dynamics and pathogenicity differ between clinical stability and periods of acute exacerbation. The association between *Prevotella melaninogenica* and clinical outcomes seen here and reported elsewhere warrants further investigation.

In this study, amplicon sequence variants rather than operational taxonomic unit clustering were employed to assign taxonomy and we were therefore able to identify *Prevotella melaninogenica* as the *Prevotella* genus member within the core microbiota. The greater resolution provided by ASV techniques offer self-evident benefit in this context given the variable outcomes associated with the genus *Prevotella* mentioned previously. ASVs also offer two further benefits over a clustered OTU-based approach: firstly, ASVs can be reproducible across studies making cross-study comparisons or even meta-analyses more consistent, and secondly, they allow improved identification of contaminants, particularly in variable biomass settings. [120,358]

A further important methodological consideration of this study is the use of estimated total abundance rather than relative abundance. Relative abundance has previously been shown to be well correlated to qPCR estimated load, [149,161] and indeed a positive correlation between overall reads and qPCR measured bacterial load were observed here. However, using relative abundance as an outcome measure in longitudinal studies or treatment-effect comparison is problematic given increases in relative abundance of one ASV must result in decreased relative abundance of other ASVs, regardless of whether the true abundance has changed. Here, total abundance was estimated by multiplying the relative abundance of each ASV by the total 16S copies (determined by 16S rRNA gene qPCR) in its respective sample, as previously described. [342,354] Given the number of 16S copies within a bacterial genome can vary from one to fifteen, abundance was also normalised to 16S copy count prior to this calculation. [359] The advantage of this approach was apparent when considering the effect of each treatment on *g_Pseudomonas_ASV001*, where AZLI+IV was associated with reduced relative abundance and IV+IV was associated with increased relative abundance. Total abundance analysis revealed both treatment effects were overstated and abundances were instead quite stable, with the apparent relative changes likely related to changes on total abundance of other taxa. Without estimating total abundance, the results in this study may have been misleading.

Recently, it has been suggested by Hahn and colleagues that clinical outcomes and changes in lung microbiota are related to antibiotic serum concentration, which can vary considerably between patients, and given antibiotic serum or sputum concentrations were not measured we cannot rule out sub-therapeutic antibiotic concentrations as a confounder. [360,361] Although sputum or serum concentrations of antimicrobials were not measured in this study, inhaled antibiotics are known to result in higher airway concentrations than their intravenous equivalents [204–206] and given the favourable clinical outcomes associated with AZLI+IV, this study would support Hahn and colleagues’ main finding that higher antibiotic concentrations are important for clinical outcomes of CF exacerbations. [361] However, our finding of more pronounced changes in the IV+IV arm differ from Hahn’s analyses. The difference in results may be partially explained by the paediatric cohort used in Hahn’s studies, where alpha diversity was higher at baseline than seen in our cohort. However, perhaps more importantly, the two groups in Hahn’s study (therapeutic antibiotic concentration group and sub-therapeutic antibiotic concentration group) were actually significantly different at baseline in terms of age, lung function, alpha and beta-diversity, making it difficult to attribute the differing antimicrobial effects solely to antibiotic concentration. The cross-over design employed here, negates some of those issues and strengthens the veracity of the findings.

Our finding that total g_*Pseudomonas*_ASV001 abundance was not reduced in either treatment arm is interesting since both arms targeted *P. aeruginosa* with aggressive doses of two anti-pseudomonal antibiotics, an approach that is considered standard practice in this setting. This finding is at odds with some previous microbiome studies where *Pseudomonas* relative abundance was reported to increase, [160,356] or in some cases decrease, [161,356] in response to treatment of an acute pulmonary exacerbation. These studies often relied solely on relative abundance and hence the limitations described earlier are applicable in that regard. Studies with rigorously quantified *P. aeruginosa* before and after treatment of an acute exacerbation have

reported no consistent effects [312,313,362,363], in keeping with the results seen here and in other microbiome studies. [130,150,354]

The lack of effect on *Pseudomonas* abundance raises questions as to the purpose of high-dose antipseudomonal antimicrobials. However, interestingly where *Pseudomonas* abundance was reduced, it was associated with better quality of life. This was not true for any other taxa, reaffirming the importance of *P. aeruginosa* in outcomes of exacerbations of CF. The methodological limitations of the sequencing approach employed here must also be considered. Firstly, despite using an ASV-based approach, sequencing of the 16S rRNA gene here was only able to resolve the dominant taxon to the genus *Pseudomonas* level at best, limiting conclusions that can be drawn without species level resolution. Second, even with species level resolution, amplicon sequencing would be unable to detect the within-species changes in phenotype and genotype previously reported during treatment of acute pulmonary exacerbations. [314] That is to say that acute, intense antipseudomonal antimicrobial therapy may impact the genotypic and phenotypic make-up of *P. aeruginosa* infection in the lung, without affecting the overall load. Such changes are not detectable by the methods used here. Metagenomic or “multi-omics” approaches, which are now becoming more widely available, will be important for investigating this possibility in the future. Finally, no attempt was made to discriminate between DNA from alive and dead bacteria. Some previous studies have utilised agents such as propidium monoazide (PMA) to bind DNA in cells with damaged external membranes (presumed to be dead), excluding that DNA from PCR amplification, sequencing and downstream analyses. [150,170,171] The decision not to use PMA was made on a number of grounds. Firstly, due to the lack of 24-hour laboratory availability at Liverpool Heart & Chest Hospital, standardising time from sample collection to PMA treatment would have been impossible. This may have introduced bias, for example cells that were viable at the time of sputum collection becoming non-viable by the time of PMA treatment. Secondly, it is not known how long DNA from dead cells persists in the lung, it is likely to vary between patients and even within patients based on their mucociliary clearance and hence adding PMA

may introduce further bias in longitudinal sampling studies such as this. Reassuringly, PMA has recently been shown to have a greater impact on the community analysis of rarer (<1% abundance) taxa rather than the core microbiota, suggesting findings here are unlikely significantly biased by the possible inclusion of non-viable DNA. [170] No difference was found between treatments in terms of changes in viable (culturable) bacterial load or total bacterial load measured by qPCR, see Chapter 5, which suggests that if non-viable DNA did mask a treatment effect in this study it would have been limited to non-culturable bacteria, for example anaerobes or dormant aerobes.

The AZTEC-CF study excluded people with CF who were not infected with *P. aeruginosa* and the results seen here, both in terms of community composition and treatment effects may therefore not be generalisable to the wider CF population. Other limitations to this study include the small sample size of only 16 participants from a single centre, which further limit the generalisability of these findings. The sample size for all comparative analyses was limited to 10 participants, such that all participants had a Day 1 and Day 14 samples available for analysis for both treatments. Despite this reduction in size, the inclusion of 40 samples for comparative analysis make this study one of the largest analyses of the CF lung microbiota in a controlled clinical trial to date. Finally, to safely conduct the AZTEC-CF study, all participants were given a test dose of AZLI during the enrolment process. The impact of a single dose, temporally dissociated from the study time-period, on the lung microbiota is unclear, but we cannot completely rule out an impact on the treatment effects we observed.

The strengths of this study lie in the cross-over design, which allowed inclusion of matched samples before and after each treatment. This approach facilitated a rigorous evaluation of longitudinal treatment effects, with each person acting as their own control, thereby minimising bias from high inter-individual variability seen here and previously reported in the wider CF microbiome literature. Other important methodological strengths include the use of estimated total abundance rather than

relative abundance to robustly assess treatment effect and ASV-based approached to allow better taxonomic resolution.

In conclusion, AZLI+IV and IV+IV exerted different effects of the lung microbiota. which appeared to be driven by reductions in total abundance of the anaerobes *Prevotella melaninogenica* and *Veillonella dispar* seen with IV+IV but not AZLI+IV. Although neither treatment consistently changed the abundance of *Pseudomonas*, when it was reduced it was associated with improved quality of life.

Chapter 7: General Discussion

The purpose of this thesis was to investigate the clinical and microbiological outcomes of AZLI in the treatment of acute pulmonary exacerbations of CF and the AZTEC-CF study was designed to evaluate these in a prospective clinical trial. The main findings are discussed and contextualised below, alongside their limitations and wider implications.

7.1 AZLI can be effective and safe in the treatment of acute pulmonary exacerbations of cystic fibrosis

Inhaled antibiotics deliver a high dose of antibiotic directly to the lung with minimal systemic exposure but as yet have been relatively untested in the acute setting. The AZTEC-CF study showed AZLI+IV may be superior to the current standard treatment IV+IV, in terms of improved lung function and quality of life, with a similar safety profile. There was no difference in the modulation of systemic inflammatory markers or time to next exacerbation, although the small study size may limit interpretation of these secondary outcomes. Overall these findings show that substituting AZLI for an intravenous antibiotic can be a safe and effective strategy in the treatment of acute pulmonary exacerbations of CF.

In the only other clinical trial in this setting, Al-Aloul *et al.* reported that nebulised tobramycin (TNS) was associated with a 3.6% improvement in lung function compared to IV tobramycin which, although not statistically significant, was not dissimilar to the +4.6% improvement seen here for AZLI+IV over IV+IV. [251] Al-Aloul *et al* did report a significantly longer time to next exacerbation for TNS, an effect not seen with AZLI. Participants in the TNS study were of similar demographics to the AZTEC-CF study and time to next exacerbation in the IV arms of each study is almost identical, suggesting that the difference between studies might be agent-specific rather than a class effect. For example, the sputum half-life of TNS is approximately 5 times that of AZLI, which could in theory play a role in the difference in longer-term outcomes. [206]

Despite the absence of any improvement in time to next exacerbation in for AZI+IV, the 4.6% improvement in lung function over IV+IV may be clinically important for two reasons. Firstly, a major concern for CF clinicians and people with CF alike is that failure to recover lung function after acute pulmonary exacerbations treated with IV antibiotics is associated with a poorer long-term prognosis. [299,300] Thus, any lung function improvement above and beyond that achieved with standard care is important. Secondly, the successful recovery of lung function means there exists potential to reduce treatment burden and systemic complications with the inhaled route.[364]

The main limitations of the AZTEC-CF study relate to the small sample size at a single centre and the unblinded nature of the interventions. The findings are therefore unlikely to instigate widespread change of practice in themselves but will inform future studies in this area. The results of the nationwide survey reported in chapter 2 showed that some UK clinicians are already adopting this approach and its use is likely to increase with time. In that regard the AZTEC-CF study taken together with the earlier TNS study provides reassurance that an inhalational approach is safe and can be effective, but also highlights the need for more research in this area.

7.2 Neither AZLI+IV nor IV+IV reduced sputum *P. aeruginosa* load

Chapter 5 and 6 addressed the microbiological outcomes of the AZTEC-CF study using both quantitative culture and culture-independent methods. Despite both treatments consisting of high dose anti-pseudomonal antibiotics, neither had a consistent effect against either *P. aeruginosa* load (by culture) or sequenced abundance (culture-independent). Although this finding was similar across platforms, improving its validity, it must be interpreted with a degree of caution given AZTEC-CF was not specifically powered towards detecting differences in bacterial abundance. In the wider literature, some studies have previously reported differences in bacterial load at exacerbations, [309,310] yet in keeping with the

findings here many recent studies, often using more robust quantification techniques, have found no consistent changes in bacterial load. [130,311,313,314]

Although no overall reductions in *P. aeruginosa* were found, there were reductions of >1 Log in nearly half of study participants, in keeping with the study by Lam and colleagues. [311] That study also demonstrated that changes in *P. aeruginosa* load did not relate to recovery of lung function during exacerbations, a finding replicated in this study.

Together, these findings could support the notion that within-population shifts in the phenotypic make-up of a *P. aeruginosa* population might be more important than overall abundance/load alone in the pathophysiology and resolution of acute exacerbations. [365] The absence of a consistent effect of either treatment on *P. aeruginosa* load despite clear clinical improvements highlights its inadequacy as an outcome measure in future clinical trials in the acute exacerbation setting.

7.3 Quantitative changes in *Pseudomonas aeruginosa* correlate with changes in quality of life

The apparent lack of a consistent effect for either treatment against *P. aeruginosa*, coupled with a lack of an obvious relationship between changes in pseudomonal load and lung function, challenges the conventional wisdom of using high dose anti-pseudomonal agents to treat acute pulmonary exacerbations. However, the finding that reductions in *P. aeruginosa* abundance alone were associated with improved quality of life reinforces the relevance of *P. aeruginosa* and reaffirms the rationale of the conventional approach to some extent. Importantly, this finding was consistent across sequencing and culture-derived quantification methods.

Baseline *Prevotella melaninogenica* abundance appeared to be associated with subsequent changes in lung function outcome and interestingly was one of the only taxa found to be differentially affected by IV+IV and AZLI+IV. A recent international collaborative sequencing study identified *Prevotella melaninogenica* as being

associated with improved lung function in the clinically stable CF setting and interestingly a potential trend towards shorter time to next exacerbation in those with a reduced post-treatment abundance was observed here. [352] The lack of relationship between any other taxa and clinical outcomes may also be an indication that the wider microbiota composition is unlikely to act as a useful biomarker by which to predict treatment response and/or allow a more targeted approach. This supports the findings of the CFMATTERS study, which remains unpublished at the time of writing but has reported no benefit for adding microbiome targeted therapy to standard exacerbation treatments. [366]

7.4 Aztreonam resistance increased with IV+IV but not AZLI+IV

Prior exposure to AZLI was an exclusion criterion of the AZTEC-CF study, and no participants had received IV aztreonam in at least five years prior to study entry. It was therefore surprising to find that half the study participants had *in-vitro* resistance to aztreonam at enrolment. Analysis of prior antibiotic exposure showed that baseline resistance to aztreonam was associated with increased meropenem exposure, but not overall antibiotic exposure, in the previous five years. This finding suggests that collateral resistance may be a driver of aztreonam-resistance in this study population.

In keeping with prior work, *in-vitro* resistance did not appear to relate to clinical outcomes but interestingly aztreonam resistance increased in the IV+IV treatment arm but not in the AZLI+IV arm. [194,204] This unexpected finding further supports the notion that collateral resistance contributes to *in-vitro* aztreonam resistance, speculatively through mutations impacting on the MexAB-OprM efflux pump or the AmpC beta-lactamase. [367] Interestingly, increased aztreonam resistance after IV+IV was seen exclusively in those with prior aztreonam resistance, while those with no resistance at baseline remained sensitive. Taken together these results suggest intravenous antibiotic use is not the most likely primary mode of acquisition of collateral resistance but does contribute to increased collateral resistance where

standing levels of genetic variation in the *P. aeruginosa* population mean that it is already present.

The main limitation of this aspect of the study is that resistance testing was performed for aztreonam only and measured relatively simplistically as cultured load in the presence of the reference MIC. Aztreonam-resistance was originally included as an exploratory safety outcome and more sophisticated techniques were therefore not included *a priori*. The finding of no increased aztreonam-resistance is reassuring in that regard, but the findings in the IV+IV arm mean more work is required to understand the mechanisms of collateral resistance to aztreonam, to determine whether collateral resistance to other even unrelated antibiotics occurs, and to determine if there are any clinical implications.

7.5 AZLI+IV and IV+IV exert different effects on the lung microbiota

In concordance with most prior microbiome work in adults with CF, the lung microbiota seen here was dominated by *Pseudomonas* but frequently enriched with anaerobes from the *Prevotella* and *Veillonella* genera. [130,146,147,150] Alpha diversity was reduced in those participants harbouring the Liverpool Epidemic Strain of *P. aeruginosa* but, contrary to a number of earlier studies, there was no relationship between alpha diversity and age or lung function. [130,133,147,153] The small sample-size of only sixteen samples for these calculations, limited to one patient per sample to avoid repeated measures bias, may have limited the ability to detect a relationship in that regard. Further studies may elucidate whether the Liverpool Epidemic Strain is an independent driver of reduced diversity, putatively by out-competing other taxa, or whether the changes seen here relate to the increased antibiotic usage and generally older age-group seen in that cohort. [286]

When comparing treatment effects, alpha diversity measures were stable across the study with no treatment-effect seen, supporting previous work suggesting alpha diversity is resilient to perturbation at both time of exacerbation and in response to treatment. [149,150] Comparing beta-diversity before and after each treatment did

reveal treatment-specific changes and the most striking changes to individual taxa were an effect against *Prevotella melaninogenica* and *Veillonella dispar* for IV+IV but not AZLI+IV. Neither treatment exerted an effect on *Pseudomonas* abundance. Speculatively, the differences in effect against the anaerobes *Prevotella melaninogenica* and *Veillonella dispar* may relate to poor penetration of AZLI into unventilated, mucus-plugged airways where anaerobes may be localised. Such areas would still receive systemic blood supply and IV antibiotics could therefore have an effect. Alternatively, colistimethate could have had a synergistic action against anaerobes in the IV+IV but not AZLI+IV treatment. No studies have been performed investigating the effect of colistimethate on the human CF lung microbiota, but a study in a sheep model found that daily IV colistimethate did not significantly influence the lung microbiota, making IV+IV synergism an unlikely explanation for these findings. [287]

This is the first prospective clinical trial to assess the impact of antibiotics on the lung microbiota and it included a number of methodological strengths, allowing a robust estimation of treatment effects. Firstly, the cross-over design allowed each patient to act as their own control thereby minimising inter-individual variation as a confounder. In addition, the use of positive and negative controls allowed a rigorous assessment of contamination or bias introduced in laboratory or sequencing processes. Finally, the study showed that relative abundance is inherently flawed as an outcome measure. Based upon these findings, calculation of total abundance in all microbiome studies attempting to estimate a causal treatment effect is recommended.

7.6 Future directions:

7.6.1 Optimising and expanding the use of inhaled antibiotics in acute exacerbations

There are a number of further research avenues indicated by the results in this thesis. Firstly, although the results of AZTEC-CF demonstrate that inhaled antibiotics can be

effective in the acute setting, all participants were mandated to remain inpatients for the treatment of their exacerbations. For the largest improvements in treatment burden to be realised, inhaled antibiotics could be used in the home setting, negating the need for inpatient admission. This will require further studies testing the efficacy of this approach, but also including assessments of treatment burden and patient preference, which were not included in this study.

There has been a recent expansion in available inhalational antibiotics, with levofloxacin inhalation solution (LIS) now licenced in the UK and nebulised liposomal amikacin having also passed through phase 3 clinical trials. [242,368] Additionally a fosfomycin/tobramycin combination is in development for CF and an amikacin/fosfomycin combination product was developed for ventilator associated pneumonia. [244,268] Elsewhere, inhaled liposomal ciprofloxacin was found to be safe and effective in non-CF bronchiectasis. [256,369] None of these preparations have been tested in the acute setting, and they may represent potential new therapeutic agents for acute exacerbations of CF and other suppurative lung diseases.

In the era of highly effective CFTR modulator therapy, exacerbation frequencies are likely to reduce and people with CF will attend hospital less frequently. Nevertheless, a residual exacerbation rate will persist and optimising the outcomes of these exacerbations while minimising treatment burden will become even more important as people with CF live with a greater degree of normalcy. Inhaled antibiotics may be one approach that allows a more ambulatory approach to future exacerbation in people receiving CFTR modulator therapy.

7.6.2 Application of AZTEC-CF results to the design of larger clinical trials

Given the pilot nature of this study, results from the AZTEC-CF study will inform design of larger clinical trials. Sample size estimations for a number of possible study designs are presented below. For all calculations an alpha of 0.05, beta of 0.2 were utilised. Based on AZTEC-CF study results, a population SD of 8.4 was included in

calculations and pre-randomisation attrition was considered as 15% for all studies. For cross-over studies between-treatment retention was considered as 75%.

Trial A: Cross-over study powered to superiority in absolute change in % predicted FEV1 between AZLI+IV and IV+IV

To detect a 2.1% difference in absolute change in % predicted FEV1 (a conservative estimate, i.e. the lower 95% confidence interval of the treatment effect seen in the AZTEC-CF study) with a power of 80%, 128 participants would be required to complete the study. Allowing for attrition and retention rates, 180 participants would be an appropriate recruitment target.

Trial B: Parallel study powered to superiority in absolute change in FEV1 between AZLI+IV and IV+IV

To detect a 2.1% difference in absolute change in % predicted FEV1 (a conservative estimate, i.e. the lower 95% confidence interval of the treatment effect seen in the AZTEC-CF study) with a power of 80%, 502 participants would be required to complete the study. Allowing for attrition, 581 participants would be an appropriate recruitment target.

Trial D: Parallel study powered to non-inferiority in absolute change in FEV1 between AZLI+IV and IV+IV

If there is truly no difference between AZLI+IV and IV+IV treatments, then 620 patients would provide the trial with 80% power to detect the lower limit of a one-sided 95% confidence interval above the non-inferiority limit of -2% absolute change in % predicted FEV1. With a 15% attrition rate accounted for 713 participants would be considered an appropriate sample size.

7.6.3 Multi-omics approach

The biobanking of paired samples across two complete exacerbation/treatment cycles in a controlled clinical trial with associated clinical outcome data will allow further studies to explore changes related to treatment and exacerbations in general. Metagenomic analyses would address some of the limitations of the 16S rRNA gene sequencing analysis undertaken, e.g. improved taxonomic resolution, whilst also providing information regarding antimicrobial resistance genes or mutations. Metabolomics could integrate understanding of the downstream implications of changes within the metagenome. Both are technically feasible on the banked study samples. The costs of these approaches at the time of study design were prohibitively expensive but have reduced significantly and will continue to reduce in the coming years. Combining species-level resolution, and in some cases strain-level resolution, with functional information about gene expression related to metabolism and antimicrobial resistance may detect changes that this study was unable to elucidate.

7.6.4 Investigating the underlying mechanisms and clinical relevance of collateral resistance

A surprising result in this study was the finding of increased aztreonam resistance induced by the IV+IV treatment but not AZLI+IV. Understanding mechanisms for this finding could be addressed by further antimicrobial susceptibility testing combined with target gene analysis, e.g. qPCR of mutations/genes associated with known resistance mechanisms. Similarly, although no increased aztreonam resistance was observed for the AZLI+IV treatment, it is important to establish whether AZLI conferred collateral resistance to other antibiotics and via which mechanisms. Given the overall relationship between *in-vitro* antibiotic susceptibility and clinical outcomes in CF is not strong, exploring the relationship between the presence of specific antimicrobial resistance genes or mutations and outcomes of acute exacerbations may be more relevant.

7.7 Conclusion:

The current project investigated the use of AZLI for treatment of acute pulmonary exacerbations. The AZTEC-CF study showed AZLI+IV was effective, safe, and superior to the current standard care (IV+IV) for important clinical outcomes. Whilst the small sample size is a limitation to the study, a major strength of this study lies in its relevance to the CF community, indeed AZTEC-CF is the only currently registered clinical trial which meets the recently identified James Lind Alliance research priorities relating to acute exacerbation (Priority 8) and antibiotic related adverse events (Priority 9) [285,370]. The cross-over design allowed a robust assessment of the microbiological effect of each treatment and showed the two treatments exert different effects on the lung microbiota but there was generally little relationship between the microbiota and clinical outcomes. Instead, despite neither treatment consistently changing the abundance or sputum load of the classical CF pathogen *P. aeruginosa*, it was still found to be associated with important clinical outcomes. These findings hint at the complexity of chronic airway infection in CF and also the limitations of 16S rRNA sequencing for identifying clinically relevant changes within CF airways. Future studies, described above, will require significant sequencing and bioinformatics capacity but it is likely technological advances will improve the feasibility of such studies in the near future. Overall, inhaled antibiotic use in the acute setting is likely to increase and the results of this project will reassure clinicians in an area relatively devoid of clinical trial evidence.

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